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A CYTOLOGICAL STUDY OF CULTURES *IN VITRO* OF JENSEN'S RAT SARCOMA.

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THE appearance and behaviour of various sarcomata when cultivated *in vitro* have been studied by several observers. Carrel and Burrows (1911) describe briefly the growth *in vitro* of rat sarcoma. Lambert and Hanes (1911), and Lambert (1913), carried out a long series of interesting observations and experiments upon rat sarcoma, but this work did not include any detailed account of the cytology of the sarcoma-cells either in fixed or in living material.

As the Jensen's rat sarcoma is at present the subject of considerable experimental work, it was thought desirable to have some more precise data concerning the structure and behaviour of the cells, and the present study was undertaken with a view to supplying such information.

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Technique.—Cultures were made by the usual hanging-drop method, and were grown either in blood-serum from a sarcoma-bearing rat or in a mixture of this serum and fowl plasma. Specimens for study by dark-ground illumination were prepared by the method described by Strangeways and Canti (1926).

For most of the cytological work cultures grown in serum were employed. The serum was usually rapidly washed off with warm Ringer's solution before fixation, as otherwise the coagulated medium greatly obscured the structure of the cells and also rendered them difficult to stain. The most accurate results were obtained by fixation in 2% osmic acid or in strong Flemming's solution without acetic acid. For a study of the cytoplasmic inclusions cultures were stained with iron-hæmatoxylin and Altmann's acid fuchsin, or with Altmann's

acid fuchsin alone, or followed by aqueous methyl green. Beautiful preparations of the cell-granules were obtained by fixing in strong Flemming's solution without acetic acid, mordanting for 10 days in Müller's solution, and staining with iron-hæmatoxylin and Altmann's acid fuchsin, or with Altmann's acid fuchsin and alcoholic methyl green. Specimens fixed in acetic Zenker and stained with iron-hæmatoxylin (long method) or by Mallory's triple stain also showed the cell-granules clearly. For the demonstration of fat, the cultures were fixed in acetic alcohol and stained with iron-hæmatoxylin followed by Sudan III or Scharlach R, or were fixed in Flemming's solution without acetic acid or with 2% osmic acid and stained with ordinary hæmatoxylin; the preparations were then mounted in Farrant's medium.

I. GENERAL APPEARANCE AND BEHAVIOUR OF CULTURES.

Cells usually begin to migrate from the explanted tumour fragment after 12 to 18 hours' incubation *in vitro*, and after 24 hours' growth the original explant has become surrounded by a fairly broad zone of tissue.

The appearance of a culture of rat sarcoma depends partly upon whether the culture medium consists of serum alone or of a mixture of plasma and serum. When cultivated in serum only (Fig. 2), the cells composing the zone of growth are diffusely scattered over the surface of the cover-glass, and the explant itself shows only slight disintegration at its margin. In a medium composed of equal quantities of serum and plasma, on the other hand (Fig. 1), the plane of outwandering is not confined, as in the serum cultures, to the surface of the coverslip, but takes place in many planes. The total emigration of cells is therefore much greater in a mixture of serum and plasma than in serum alone, and as a result of this active outwandering the peripheral region of the original fragment often spreads and thins out into a compact, irregular sheet of tissue, which gradually merges into a more scattered zone of growth. These slight differences between the type of growth obtained in serum alone, and in a mixture of serum and plasma, is probably due to the mechanical support afforded to the outwandering cells by the fibrin clot of the mixture as opposed to the fluid character of the pure serum. On the other hand, the cells grown in a mixture of plasma and serum are usually healthier, and show proportionately more mitosis than those cultivated in serum alone, which would seem to suggest that it is not the mechanical factor only which renders the mixture a more favourable medium.

A typical, healthy culture of Jensen's rat sarcoma consists of two main types of cells, which are readily distinguishable from each other in both structures and behaviour. For the sake of convenience these two form will be referred to as the fibroblastic and wandering type respectively.

The character of the zone of growth depends to some extent on the relative proportions of these two cell-types. The wandering cells are the more motile, and in a serum and plasma medium are usually the first cells to crawl out from the explant. Thus during the first 18 to 24 hours of cultivation the wandering cells usually preponderate greatly in the zone of growth, which in extreme cases is almost wholly composed of these elements, the fibroblast-like cells having only just begun to emerge from the fragment. In most serum cultures this predominance of amœboid cells during early growth *in vitro* is less marked. The majority of the wandering cells appear to wander out from the explant during the first two days *in vitro*, and gradually crawl far out into

the medium, sometimes almost to the boundary of the fibrin clot. In 3 to 4-day cultures grown in serum and plasma, the zone of growth therefore usually consists of an inner region largely composed of fibroblast-like cells, and a broad, outer region occupied by scattered wandering cells.

In the serum cultures mitosis is rare and often quite absent, but in favourable cultures grown in a mixture of serum and plasma it is very abundant, especially in the neighbourhood of the explant (Figs. 3 and 5).

An interesting feature of the growth *in vitro* of sarcoma, which has already been noted by Fischer (1925) in the case of the Rous sarcoma of the fowl, is the readiness with which the cells will carry on an independent existence when isolated from their fellows. It is common to find single cells and small scattered groups of from 5 to 30 cells near the margin of the medium, remote from the original fragment. In many cases (Fig. 18) such isolated cells appear as healthy as those in the zone of growth immediately surrounding the explant, but others exhibit degenerate features. Both the wandering and the fibroblast-like cells occur among these independent elements. Many of the isolated cells have doubtless emigrated from the explant, but the majority appear to have become artificially separated from the main fragment at the time of explantation.

Cultures of rat sarcoma in chicken plasma and rat serum may remain alive for 3 to 4 weeks without change of medium. In such cultures the cells are almost entirely of the fibroblast-like type, as most of the wandering cells degenerate after about 9 days *in vitro*. Mitosis may continue active up to the sixth or seventh day of growth. Cultures in pure serum die out after 8 to 13 days *in vitro*.

II. CYTOLOGICAL STRUCTURE AND BEHAVIOUR OF THE TWO CELL TYPES.

1. *Cytological structure:* (a) *Fibroblastic type.*—Cells of the fibroblastic type are very variable in *shape*, but the majority are spindle-shaped, fan-shaped or triangular. Many, especially in serum cultures, show one or more long, slender processes, often expanded at the ends into small delicate fans of protoplasm (Fig. 17). Cells which are attached to the coverslip are usually expanded into a triangular or fan-shaped form, whilst cells lying freely in a fibrin clot tend to assume the fusiform shape.

The *size* of the fibroblast-like cells is also very variable, the largest being almost twice as large as the smallest.

The *nuclei* are oval or rounded, and contain several large and numerous small, irregular granules of chromatin (Fig. 17). In some cells these granules are larger and more abundant than in others. In cultures grown in serum and plasma, mitosis is common.

The cytology of the cytoplasm, as stated above, is studied best in cultures grown in serum only.

A *centrosphere* appears te be invariably present in the cells, at least in serum cultures, although in many cases it is extremely difficult to distinguish. In the more healthy cells it is seen as an irregularly shaped area of protoplasm with a rather distinct periphery, which usually lies on the side of the nucleus farthest from the explant; its size is about one-fifth that of the nucleus. It

is surrounded by a dense mass of cytoplasm occupied by numerous minute granules, mitochondria and a few small fat-globules. The centrosphere appears to contain two centrioles, but owing to the granular character of the surrounding cytoplasm it is difficult to be certain of the identity of the centrioles.

The *mitochondria* are filamentous or rod-shaped, and throughout the cell they tend to lie with their long axes radiating from the centrosphere (Fig. 19).

The *fat-globules*, which are small and not very numerous in healthy cells, are mostly situated in the neighbourhood of the centrosphere. They become brown when fixed with osmic acid, and stain brilliantly with Sudan III and Scharlach R after fixation with formalin or acetic alcohol (Fig. 17).

(b) *Wandering cells.*—The average *size* of the wandering cells is considerably smaller than that of the fibroblast-like elements (Fig. 18), although the largest wandering cells are about as large as medium-sized cells of the fibroblastic type.

As the wandering cells emerge from the explant they assume extraordinarily irregular *shapes*, frequently exhibiting a complicated arborescent form somewhat reminiscent of a nerve-cell with dendrites. This alteration in shape during emigration from the explant is beautifully seen in certain cultures grown in plasma and serum, in which the outer part of the explant has spread over the coverslip as a thin, continuous sheet of tissue before breaking up into the scattered cells which eventually form the greater part of the zone of growth (Fig. 5). In such specimens the inner regions of the continuous sheet is composed of cells which, although very variable in size, are of comparatively regular outline, the majority being approximately oval, round or fusiform, so that the general appearance is that of an ordinary histological section of the sarcoma. Towards the periphery of the sheet the cells become more expanded and less regular in shape, and at the actual margin the wandering cells put forth numerous lobed and branched pseudopodia, usually in several planes. These pseudopodia are for the most part directed away from the explant, so that cells are seen which on the side nearest to the explant have a smooth, regular contour, but on the further side are produced into long, complicated pseudopodia. The contrast between the wandering cell and the adjacent fibroblast-like elements is most striking in such a preparation, since the larger fibroblast-like cell usually protrudes only 1 to 3 processes of a simple tongue-shaped or elongated form. As the wandering cells crawl further into the medium they become still more irregular in form, although the long branched processes continue to be directed along the line of migration, *i. e.* away from the explant, whilst the part lying towards the explant remains comparatively smooth (Fig. 4).

The *nucleus* of the wandering cell is usually highly irregular in shape, and shows lobes protruding into the cytoplasmic branches (Fig. 4). These lobes are sometimes connected with the rest of the nucleus merely by a slender, almost imperceptible neck, so that the cell looks as if it were about to divide by amitosis. The nucleus contains numerous irregular granules of chromatin of different sizes.

In cultures grown in a mixture of serum and plasma *mitosis* is common among the wandering cells. Sometimes, especially in the neighbourhood of the explant, the wandering cells round up into an oval or spherical form before

beginning division. It is equally common, however, to find the various mitotic phases present in cells which show the wildest irregularity of outline (Fig. 4).

The wandering cells usually, if not invariably, contain a number of *cytoplasmic granules* of different sizes and staining capacity (Fig. 18). By adding solid carmine to the medium in which the cells are cultivated it is possible, as previously described by Lambert and Hanes, to obtain specimens in which these granules are beautifully and permanently stained *intra vitam* (Fig. 20). The actual ingestion by the cell of solid particles of carmine does not appear to be essential for this result, as brightly stained granules are seen in cells which contain no solid carmine.

As in the fibroblast-like cells, there is a small irregular *centrosphere* on one side of the nucleus around which are aggregated numerous granules and filamentous and rod-shaped *mitochondria*; other mitochondria are scattered throughout the cell (Fig. 18). The average length of the mitochondria in the wandering cells is usually rather shorter than in cells of the fibroblastic type.

Fat-globules staining vividly with Sudan III (Fig. 17) and Scharlach R are always more abundant in the wandering cells than in the fibroblast-like elements. The majority of the globules are massed in the more regular-shaped part of the cell lying towards the explant.

2. *Appearance and behaviour of the living cells:*—(a) *Direct illumination: fibroblast-like cells.*—The internal structure of the cells is not well shown by direct illumination. The cytoplasm of the fibroblast-like elements appears clear and homogeneous and the outline is not very clearly seen. The oval nucleus is fairly distinct, and is seen to contain one or more large and numerous smaller greyish granules. It is possible to distinguish faintly the granular condensation of cytoplasm lying to one side of the nucleus, and bordering this area the zone of small fat-globules, which are brilliantly refractile.

Mitosis is readily observed on the warm stage (Fig. 11). The cell which is about to divide withdraws its processes in the usual way and becomes rounded or pear-shaped. The prophase filaments become arranged at the equator of the cell in the metaphase plate. The chromosomes of the equatorial plate can be seen to grow somewhat shorter and stouter, and then quite suddenly split longitudinally. The sets of daughter-chromosomes thus formed pull apart and pass fairly rapidly to the poles of the cell, a rapidly narrowing constriction appears at the equator of the cytoplasm, whilst the surface of the cell shows the "bubbling" described by Strangeways (1922) in the case of the normal mesenchyme cell, and in the course of a few minutes the original cell is nipped into two daughter-cells. Each of the two groups of chromosomes has meanwhile condensed into a small, oblong, greyish nucleus. The two daughter-cells enlarge, put forth pseudopodia and spread out over the surface of the coverslip, whilst their somewhat opaque nuclei enlarge into oval vesicles containing a dense network of faint grey filaments. This dense network of filaments becomes progressively more diffuse and irregular, and eventually the nucleus appears as a large, clear oval or spherical body containing numerous granules and irregular rods of various sizes. By this time the outline of the daughter-cells is similar to that of the fully formed neighbouring elements.

Wandering cells.—The wandering cells, as already described in the case of fixed specimens, are much more irregular in outline than the fibroblast-like cells and contain more fat-globules. Lying amongst the fat-globules and somewhat obscured by them are the cell-granules, some of which can just be distinguished as faint, greyish spheres. They are most readily studied when stained *intra vitam* either with a dilute solution of neutral red, as described by Lewis and Gey in the case of the mouse sarcoma, or with carmine in the manner to which reference has already been made

(b) *By dark-ground illumination: fibroblast-like cells.*—For a study of the cytology of the living cell the method of dark-ground illumination is admirable. The close correspondence between the structure of the cell as demonstrated in living material by this technique and as shown by the ordinary cytological methods employed in the preparation of fixed and stained specimens is most encouraging to the cytologist.

The structure of the fibroblast-like cells (Fig. 6) is studied best in cells which are spread out on the surface of the coverslip as thin sheets. Such a cell is usually slightly elongated in the direction of migration, and the outline appears as a thin white line, which in places, especially around the ends of the pseudopodia, is produced into delicate needle-like processes adherent to the glass.

The nucleus is seen as an oval, dark area bounded by a white line, and containing numerous irregular rings of variable size which represent the chromatin granules.

On the side of the nucleus most remote from the explant lies the dense area of protoplasm surrounding the centrosphere, which by dark-ground illumination appears as a mass of fine whitish granules; the centrosphere itself is not seen. The tract of small fat-droplets by which this mass is usually bordered is very conspicuous owing to the high refractive index of the fat-globules, which are the most brilliant objects in the cell. As in the fixed specimens, a large number of mitochondria are usually aggregated in the neighbourhood of the centrosphere, whilst others are distributed throughout the cytoplasm, their long axes tending to radiate from the centrosphere. The mitochrondria appear as whitish filaments and rods, which are finer and less refractile than those of normal mesenchyme cells illuminated by the dark-ground method. Numerous small whitish granules are scattered throughout the cell.

When the cells are observed at body temperature they are seen to be in slow but constant movement. The outline of the cell is gradually but continually changing. Large elongated or wedge-shaped pseudopodia are very slowly protruded, and may be quite suddenly withdrawn, leaving behind numerous long, needle-like processes of cytoplasm, which also are eventually retracted into the cell-body. The cell may gradually expand or contract and small fluctuations in contour are perpetually seen. The final result of these surface changes is movement of the entire cell away from the explant. Within the cell, especially in the pseudopodia, the cell-organs show considerable motility. The long, filamentous mitochondria pass slowly to and fro, twisting and bending and sometimes breaking into two, whilst the smaller rod-like forms move rather more rapidly. The fat-globules usually oscillate slightly and now and then

shift their position in the cytoplasm. The small cell-granules are perhaps the most motile constituents of the cell and dart actively about. Very little movement is observed in the dense mass of granular protoplasm lying near the nucleus.

Wandering cells.—The highly branched condition of the wandering cells is beautifully shown by dark-ground illumination. The internal structure is also very well displayed, and the nucleus with its chromatin granules, the dense granular area surrounding the centrosphere, the rod-like and filamentous mitochondria and small cell-granules are readily distinguished (Fig. 8). As in fixed material, the greater part of the fat is concentrated on the side of the nucleus nearest the explant, but large groups of globules occur elsewhere. The larger cell-granules which, as described above, are so conspicuous when stained *intra vitam* with neutral red or carmine, can be seen as white rings which are rather more brilliant than the mitochondria, but less so than the fat-globules.

The wandering cells present a most striking contrast to the fibroblast-like elements in the remarkable activity of their movements. Pseudopodia are thrown out, with surprising rapidity, glide, swell and are withdrawn as rapidly, to start anew from a fresh site in the surface of the cytoplasm. The nucleus is often correspondingly distorted, and lobes, sometimes showing a marked constriction, bulge into the bases of the main pseudopodial branches, only to be drawn back into the main body of the nucleus as the pseudopodia are retracted. Some idea of the constant alteration in shape exhibited by both nucleus and cytoplasm is obtained from the set of serial drawings shown in Fig. 9, in which the sketches were made at intervals of one minute. The restless turmoil of the cell is enhanced by the violent activity of the inclusions. Mitochondria, small fat-globules and cell-granules scour to and fro along the pseudopodia, jostling one another, and showing at the same time a ceaseless, dancing motion resembling Brownian movement. The granular mass of protoplasm surrounding the centrosphere, around which many of the mitochondria are aggregated, is also the site of intense agitation which reminds the observer of the seething movement of a swarm of bees. When a new pseudopodium is protruded in the neighbourhood of a large group of fat-droplets, it carries the fat with it in a stream of brilliant, dancing globules, which make a rather striking sight. In some cultures made for dark-ground illumination a sheet of tissue is seen similar to the sheet already described in the case of fixed preparations. In such specimens certain of the wandering cells are still of comparatively regular spindle or rounded form, but may nevertheless be distinguished from the neighbouring fibroblast-like elements by the active motion of their cell inclusions. Continued observation of such a wandering cell shows that the regularity of outline is transitory, and the cell soon begins to throw out the characteristic pseudopodia described above.

3. *Phagocytosis.*—As already described by Lambert and Hanes, the wandering cells in cultures of Jensen's rat sarcoma are extremely phagocytic, and if carmine particles are added to the culture medium many of these cells become loaded with these granules. The fibroblast-like cells also take up the carmine, but less readily than do the wandering cells.

Both types of cell show a strong tendency to ingest cell *débris*, and may

even envelope an entire degenerate rounded cell which lies in a vacuole in the cytoplasm, where it appears to be digested.

III. ABNORMALITIES AND DEGENERATIVE PHENOMENA.

1. *Multinucleate and binucleate cells.*—In the majority of cultures large binucleate and multinucleate cells of the fibroblast-like type occur, and in some specimens are quite numerous.

Binucleate cells may be spindle-shaped or flattened on the coverslip in a triangular or polyhedral form. A certain proportion if not all of these cells are formed by abnormal mitosis, as recorded by Lambert and Hanes, and Strangeways (1924). An interesting example of this incomplete division was observed by the present writers in a culture which had really been prepared for study by dark-ground illumination, and which had been growing for five days in unchanged medium. Noting that mitosis was present and suspecting that much of it would be abnormal, the culture was observed by means of ordinary direct illumination. The cell selected for study was well isolated from its neighbours, of spherical shape, and contained a very distinct metaphase plate and numerous fat-globules (Fig. 12). Observation began at 3.7 p.m. Three minutes later the chromosomes had split into two groups, which had drawn apart into a typical anaphase. It was not until 3.20, however, that the cytoplasm began to show any constriction—an unusually long delay between anaphase and telophase. By 3.24 the constriction was reduced to a narrow isthmus, which, however, did not break in the usual way, but slowly became drawn out into a delicate, almost imperceptible thread of cytoplasm—its condition at 4.7. Meanwhile the two groups of chromosomes had each given rise in the usual way to a typical daughter-nucleus, whilst the fat-globules had become distributed in two large groups, one in either half of the cell. Further change was not observed until 4.25, when it was found that the fine connecting thread of cytoplasm was gradually growing shorter and thicker. By 4.43 this thread had expanded into a thick neck, at 4.50 it was represented by a mere constriction and by 5.25 it was no longer discernible, and the two halves of the cell had completely merged. The two groups of fat-globules flowed together to form a single aggregation in the middle of the cell between the nuclei. Meanwhile the cytoplasm had been gradually elongating, and by 7.10 the cell appeared as a typical, fusiform, binucleate element. Binucleate individuals also occur among the wandering cells, but are comparatively rare.

The multinucleate cells appear to be exclusively of the fibroblast-like type (Fig. 13). They may be fusiform or polyhedral in shape, and contain from four to fifty nuclei, which in a single cell often range in size from minute globules to normal-sized structures. The total volume of nuclear material in one of these atypical cells is usually very greatly in excess of the normal amount. The nuclei are almost always distributed in a ring about the centrosphere. Both large and small nuclei have essentially the same structure, *i.e.* they consist of one or more large chromatin granules and numerous small particles scattered in a colourless ground-substance. The writers are unable to indicate how these multinucleate cells arise, as their formation has not been observed in a living culture.

2. *Enlarged centrospheres.*—Enlarged centrospheres similar to those described by Lewis (1920) in degenerating mesenchyme cells and by one of the writers (Fell, 1925) in hypertrophied chondroblasts are common in serum cultures of J.R.S. after 3 or 4 days' incubation. The enlarged centrospheres were observed in the fibroblast-like cells only. As previously stated, the centrosphere seems to be a normal constituent of the fibroblast-like elements, and consists of an irregularly shaped area on one side of the nucleus. This area, in Zenker-fixed material stained with Grübler's eosin-methylene blue, is seen to have an oxyphil interior with a basophil periphery. In certain cells of old cultures the original centrosphere is seen to be abnormally large, and is surrounded with a well-marked oxyphil halo with a basophil border; this halo is bounded externally by a zone of fat-globules, and apparently corresponds with the "cortical zone" of the giant centrospheres described by Lewis. In preparations fixed in osmic acid and stained for mitochondria, the cortical zone is seen to be occupied by a cloud of minute, lightly staining granules and small radiating mitochondria. In some cases the outline of the cortical zone is vague so that the centrosphere fades gradually into the general cytoplasm, but in other cells, which appear to be more degenerate, the centrosphere is quite sharply defined. The maximum size attained by the centrosphere may considerably exceed that of the nucleus, and in certain cells the medullary region (original centrosphere) alone is as large as the nucleus.

3. *Epithelioid cells.*—In some serum cultures the zone of growth consists mainly of a type of cell which differs from either the fibroblast-like or the wandering elements (Figs. 14 and 22). When fibroblastic cells are present in such cultures they usually exhibit degenerative features. As this third type of cell shows many degenerative characteristics and are most numerous in what appear to be unhealthy cultures, it was thought that an account of their cytology should be included in the present section.

The average size of these cells is intermediate between that of the normal fibroblast-like cells and that of the wandering cells. In shape they are usually oval, elongated, triangular or fan-shaped, whilst a minority are branched and irregular. The cell margin in fixed preparations has in places a curious bloated, almost cystic appearance, whilst elsewhere it appears thin and sheet-like. As shown by dark-ground illumination this edge in the living condition is in a state of perpetual slow movement, becoming thick and refractile in one place, thin and faint in another, at one point temporarily detaching itself from the glass and curling upon itself or undulating freely in the medium, whilst everywhere the general outline of the cell shows continuous but shallow fluctuations.

The nucleus is considerably smaller than in the fibroblastic cells. It is spherical or oval and contains one or more nucleoli. Mitosis was never observed.

The cytoplasm is usually sharply divided by a zone of fat-globules into two regions—an inner and outer region. The inner region, which is the larger of the two, is crowded with cell-granules and a smaller number of fat-globules and clear vacuoles. In specimens stained *intra vitam* with neutral red, the granules of a single cell show great variation in staining reaction, some being

rapidly coloured a bright red, whilst others are hardly coloured at all, even after a fairly prolonged contact with the stain. They exhibit a similar variation in their reaction to cytological stains in fixed material, and in the same cell display every grade of coloration from extreme basophil to extreme oxyphil; this is particularly well shown in specimens fixed in strong Flemming's solution without acetic acid, mordanted for some days in Müller's solution, and stained either with iron-hæmatoxylin and acid fuchsin, or with acid fuchsin and alcoholic methyl green (Fig. 22). The granules are very variable in size but are of a regular spherical form. Vacuoles of an irregular shape which have similar staining reactions as the cell-granules, both when stained *intra vitam* with neutral red and in fixed material, also occur quite commonly. A centrosphere lies to one side of the nucleus and can be distinguished as an irregular area of rather dense protoplasm; it is so obscured by the cell-granules that its precise structure could not be determined.

In the outer region of the cytoplasm the granules are rare and often absent. The mitochondria, on the other hand, are very distinct in this area, and are seen to consist of granules and rather stout rods of different length (Fig. 14); they are not nearly so numerous as in the fibroblast-like cells. The ground-substance of the outer region of cytoplasm has a curious hyaline appearance very unlike that of the fibroblastic cells. In specimens fixed in osmic acid or Flemming's solution without acetic acid the mitochondria stand up sharply against this clear background (Fig. 14), which with a modification of Mallory's triple stain assumes a beautiful sky-blue shade, contrasting well with the brownish mitochondria and granules.

In living cultures illuminated by the dark-ground method it is found that the movements of the various cytoplasmic inclusions are more active than in the fibroblast-like cells.

In old cultures the majority of these cells round up. The cytoplasm becomes concentrated about the nucleus until the cell appears as an oval mass of protoplasm gorged with granules and fat-globules, and protruding from its surface numerous small, crumpled, cystic and lamellar processes.

The epithelioid cells appear to be derived from the wandering cells. They seem to have no relationship to the fibroblastic elements, from which they differ in the size of the nucleus, in the appearance of the cytoplasm, in the character of the mitochondria and in the degree of cellular activity. On the other hand, every transitional stage between a typical epithelioid and a typical wandering cell can be found in many cultures. The presence of large numbers of big cell-granules and the absence of mitosis would suggest that this transformation of the wandering cells is a degenerative process.

4. *Cellular hypertrophy.*—If a serum culture is kept unchanged for about three days it will be found that a large proportion of the cells have degenerated, others are comparatively normal, whilst the remainder are greatly enlarged. Some of these enlarged cells reach their maximum size about the sixth or seventh day, when they may be anything from four to eight times their original size.

A varying proportion of these giant-cells are quite obviously formed by the enlargement of the fibroblast-like cells, from which they differ only in their

larger size (Fig. 15). This type of giant-cell usually contains from one to three nuclei. The centrosphere is very large, and is surrounded by a somewhat diffuse zone of fat-globules and a varying proportion of cell-granules; the mitochondria are very numerous and often appear quite normal.

There is a second type of giant-cell, however, the origin of which is less evident. These cells differ from the former type in the relatively smaller size of nucleus or nuclei and in the sharp division of the cytoplasm into two regions of approximately equal area. The inner region surrounding the nucleus contains a distorted centrosphere, and is occupied either by a dense mass of small fat-globules which, when the fat is dissolved out, gives this area a striking honeycombed appearance, or by an outer zone of fat-globules enclosing a mass of cell-granules. The outer zone is composed of clear, vitreous cytoplasm, which is very transparent in the living cell and difficult to stain in fixed material. The largest of the cells are roughly oval or circular, but others are irregular in shape, and are sometimes associated with enormously long rod-like tails of cytoplasm ending in a club- or fan-shaped expansion. These cells appear to be formed from the epithelioid elements described above, which they closely resemble in cytological structure; some cells are found which might be interpreted as forms intermediate between the epithelioid and giant-cells. The evidence, however, is not sufficiently conclusive to warrant a dogmatic statement on this point.

By the thirteenth day *in vitro* most of the hypertrophied cells of both types have rounded up and degenerated.

Although more common and more easily studied in cultures grown in serum alone, cellular enlargement also occurs when a mixture of serum and chicken plasma is employed as a medium.

This hypertrophy is by no means peculiar to cultures of sarcoma, but has also been observed in old serum cultures of normal embryonic connective tissue from the heart, kidney and tail of a fœtal rat.

5. *Cell death:* (a) *Fibroblast-like cells.*—In a culture which from age or for some other reason is in an unhealthy condition the fibroblast-like cells show degenerative changes. There are several types of degeneration, but the usual process, all stages of which may be observed both in fixed and in living material, is as follows:

The fat-globules surrounding the centrosphere increase in size and number, whilst just between the zone of fat and the centrosphere numerous small granules appear which stain *intra vitam* with neutral red, and in fixed preparations show a strong affinity for hæmatoxylin or acid fuchsin. Small vacuoles which do not stain with neutral red or with any of the cytological stains employed also usually appear at this stage in the zone of fat-globules, and, less commonly, in other parts of the cell. By dark-ground illumination these vacuoles are seen as clear, dark spaces, delineated by a whitish line, and are about as bright as the neutral red granules. A change is also observed in the cell margin, which shows fluctuations similar to but less pronounced than those described in the case of the epithelioid cells; this phenomenon has also been noted in ordinary mesenchyme cells in old dark-ground cultures. The nucleus shows little change, except that in the living cell the ground-substance has a faintly granular appearance when illuminated by the dark-ground method.

As degeneration proceeds the cytoplasm tends to condense around the nucleus in a densely staining mass instead of being spread sheet-like over the surface of the glass. Indentations in the fan-shaped or triangular cytoplasm appear at this stage and become deeper, until at length the cytoplasm is split up into broad, flat, radiating pseudopodia with active marginal movement at their expanded ends. The fat-globules and granules continue to increase in number, the centrosphere becomes shrunken and distorted, and many of the mitochondria break up into rods and granules; in some cells a marked diminution in the number of mitochondria is noted.

The broad, flattened pseudopodia gradually narrow into rod-like processes terminating in a small bunch of blebs and twisted lamellæ, whilst the bulk of the cytoplasm has accumulated around the nucleus to form a more or less oval cell-body.

As the cytoplasm continues to concentrate about the nucleus the long pseudopodia are gradually withdrawn, until the cell is reduced to an oval mass of protoplasm filled with granules and fat-globules. Liquefaction then sets in and a large vacuole appears which enlarges until the cell is reduced to a mere vesicle in which, in a living culture, the cytoplasmic inclusions dance in active Brownian movement.

(b) *Wandering cells.*—As recorded above, the wandering cells normally contain a fairly large quantity of fat and neutral red granules, and one of the first indications of an unhealthy condition is a marked increase in the amount of both these types of inclusions. At the same time both the amœboid and the intracellular movements become more pronounced, whilst small cystic processes are formed at different points on the surface. These small cystic structures are well seen by means of dark-ground illumination as clear, dark vesicles outlined in white. Their formation often precedes the protrusion of true pseudopodia: an irregular cyst is rapidly formed, wavers for a moment, and suddenly collapses into a small, more solid process, which then extends as an ordinary pseudopodium. Sometimes these cystic processes are comparatively flat and sheet-like, and show undulations similar to the movements seen at the periphery of the fibroblast-like cells. Short, thorn-like processes are also repeatedly put forth and withdrawn.

As the degenerative changes advance the cells become gorged with fat-globules and granules, whilst the mitochondria grow shorter and thicker, tending towards the rod-like rather than the filamentous form. The cells exhibit even greater motility than before, crawling slug-like over the more quiescent fibroblastic elements. They no longer put forth the long, arborescent pseudopodia which characterize their earlier healthy state; on the other hand, the formation of superficial cysts is more marked, and short, broad pseudopodia are still protruded.

The ultimate fate of such a cell is not always the same. Sometimes it breaks down quite rapidly into a mass of small, spherical vesicles, each containing granules and fat-globules in violent Brownian movement. At other times it dies more slowly. One such case of slow degeneration was observed in a living culture by means of dark-ground illumination during a period of 6 hours. The cell selected for study contained a considerable quantity of fat, and was protruding surface cysts and short pseudopodia (Fig. 16). Observation began

at 11.25 a.m., but no alteration was noted until about 12.35 p.m., when it was seen that the pseudopodia protruded were becoming rather longer and thinner and usually had expanded ends, whilst the cytoplasm was beginning to condense around the nucleus into a roughly spherical mass. As time passed this attenuation of the pseudopodia became more and more pronounced, so that by 1.20 the majority of the processes appeared as delicate filaments, usually but not invariably distended at the tips. Thicker pseudopodia were still sometimes formed, often by the enlargement of a filamentous process; conversely the thick pseudopodia frequently dwindled into filaments. It was interesting to watch the fat-globules, the diameter of which many times exceeded the diameter of the thread, pass freely up and down the cytoplasmic filaments, the phenomenon being somewhat reminiscent of a grass-snake swallowing a frog. At 1.40 the broad end of a pseudopodium was seen to break away from its filamentous stem and disintegrate.

From 3.30 onwards even the filaments began to disappear, being formed at progressively longer intervals. Large vesicular processes, however, began to bulge out from the surface, which were larger and more regular in contour than the small crumpled cysts of earlier stages. In the now spherical cell-body the fat-globules gradually coalesced into a smaller number of large droplets, whilst the cytoplasm became steadily more liquid, as shown by its increasing transparency and the active Brownian movement of the cell inclusions.

In the final stages of degeneration even the cystic structures ceased to be protruded. The last of these cysts to be formed was not withdrawn, and at 4.55 became detached from the shrunken main mass as a small independent vesicle. By 5.30 the cell was represented merely by a small, shrunken, round body laden with fat-globules. Observation was then discontinued.

DISCUSSION.

The results of this study illustrate certain advantages of tissue culture as a histological technique as compared with the examination of tissues by means of sections of fixed material. In cultures *in vitro* the individual cells are isolated, their cytological character, amœboid and intracellular movements, mitosis and degenerative changes can be studied in the living condition, and observations made on the fixed specimens can be checked by examination of the living tissue.

The question presents itself as to whether the two cell types observed in cultures of Jensen's rat sarcoma are histologically distinct classes of tissue, or whether the one represents an earlier developmental stage or morphological phase of the other. Lambert and Hanes regard the two forms of cell as morphological variations, not as "generically separate types," but Lewis and Gey (1923), working on a mouse sarcoma, appear to consider the active amœboid elements present in their cultures as quite distinct from the more quiescent spindle-shaped cells. That the fibroblast-like cells should transform into wandering cells seems improbable, in view of the considerably greater average size of the former type of cell as compared with the latter. In the light of recent work indicating that monocytes can develop *in vitro* into fibroblasts, it would seem more likely that the wandering cells of the sarcoma

might give rise to the fibroblast-like cells. The results of the present writer's investigations, however, afford no evidence of any histogenetical relationship between the two cell types. In fixed material, cells which might be interpreted as intermediate between the two types are quite commonly found. Such "transitional forms," however, should be accepted as such with considerable reserve, since, as already pointed out, certain wandering cells in living cultures may appear temporarily as fairly regular spindle-shaped cells, which in fixed material would have a striking resemblance to fibroblast-like elements and might easily be mistaken for intermediate forms. Dark-ground illumination of such cells, however, renders them easily distinguishable from the neighbouring fibroblast-like cells by the much more active movements of their cytoplasmic inclusions; if observed for some time these cells will be found to assume eventually the irregular shape characteristic of a typical wandering cell *in vitro*. The epithelioid elements resemble the fibroblast-like type more nearly than do the wandering cells of a healthy culture, and might at first sight be regarded as a connecting link between the two forms. It is, however, only the degenerate fibroblastic cells laden with fat-globules and granules which the epithelioid cells resemble, and even from these they differ in nuclear volume, in intracellular activity and in the appearance of the cytoplasm.

The fibroblast-like cells are very similar in structure and behaviour to embryonic connective-tissue cells from the various organs of the fœtal rat. They differ from embryonic connective tissue mainly in their more discrete habit of growth, and in the readiness with which they lead an independent existence isolated from the neighbouring cells. No important structural dissimilarities were observed, except that in an embryonic connective-tissue cell illuminated by the dark-ground method the mitochondria appear longer, stouter and more refractile than in the fibroblastic cells of sarcoma. The wandering cells are undoubtedly of lymphoid character, and by dark-ground illumination are indistinguishable in structure and behaviour from the wandering cells of normal embryonic tissue cultivated *in vitro*, or from the larger lymphoid cells in cultures of adult spleen. Lewis and Gey in their paper on mouse sarcoma regard the wandering cells as clasmatocytes—an interpretation with which the present writers entirely agree in the case of the Jensen's rat sarcoma.

The writer's observations shed no light on the question as to which of the two cell types present in Jensen's rat sarcoma is the malignant agent. Carrel (1924), working on the Rous sarcoma of the fowl, has adduced experimental evidence to show that lymphoid elements (monocytes) are responsible for the malignancy of the Rous tumour. By analogy it is possible, therefore, that the wandering cells or clasmatocytes may be the malignant components of the rat sarcoma, whilst the fibroblast-like cells represent stromal elements.

SUMMARY.

1. Cultures *in vitro* of Jensen's rat sarcoma were made, and a culture medium consisting either of serum from a sarcoma-bearing rat or of a mixture of this serum and chicken plasma was employed.

2. Cultures grown in serum alone showed less extensive outwandering,

much fewer mitotic figures, and died out more rapidly than cultures grown in a mixture of chicken plasma and rat serum.

3. Two types of cell were present in the cultures, and occurred in about equal numbers: (*a*) cells resembling fibroblasts, and (*b*) smaller wandering cells similar to clasmatocytes.

4. The cytology of the two types of cell is described. The wandering cells contained more fat-globules and cell-granules than the fibroblast-like cells, from which they also differed in the complex arborescent form which they assumed on emerging from the explant.

5. Cultures were studied on the warm stage by means of dark-ground illumination. It was found that the wandering cells were far more motile than the fibroblast-like elements, and the movements of the various cytoplasmic inclusions were also considerably more active.

6. Giant centrospheres occurred in the fibroblast-like cells of old serum cultures.

7. Binucleate cells were common. The formation of such a cell by incomplete mitotic division was observed in a living culture. Multinucleate cells containing as many as forty nuclei of very varied sizes also occurred.

8. Cells resembling epithelioid cells were abundant in certain cultures. They seemed to be formed from the wandering cells and to be of a degenerate character.

9. In old (4 to 7-day) serum cultures a certain proportion of the cells hypertrophied, sometimes enlarging to seven or eight times their original size.

10. The cytological changes preceding cell death are described.

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DESCRIPTION OF PLATES.

c.z. Cortical zone. *f.g.* Fat-globules. *fib. c.* Fibroblastic-like cell. *m.z.* Medullary zone. *mi.* Mitosis. *n.* Nucleus. *wand. c.* Wandering cell. *x.* Protoplasm detached from cell.

Note.—Figs. 17–22 are all drawn to the same scale.

Fig. 1.—Culture fixed after 48 hours' growth *in vitro* in a medium composed of equal parts of serum from a tumour bearing rat and chicken plasma. (Acid alcohol; iron-hæmatoxylin.)

Fig. 2.—Culture fixed after 48 hours' growth *in vitro* in serum from a tumour-bearing rat without the addition of chicken plasma. The growth is of a more scattered nature than in the culture shown in Fig. 1. (Acid alcohol; iron-hæmatoxylin.)

Fig. 3.—Group of cells, mainly of the fibroblast-like type, in a 48-hour culture grown in chicken plasma and serum from a sarcoma-bearing rat. (Acid alcohol; iron-hæmatoxylin.)

FIG. 4.—Group of wandering cells from the same culture. One of the cells is in mitosis. Note the highly irregular shape and small size of the cells as compared with the fibroblast-like elements shown in the previous figure.

FIG. 5.—Culture showing the outwandering of the sarcoma-cells from the explant into the culture medium. The cells lying inside the sheet of tissue are comparatively regular in shape as compared with those at the periphery. Both types of cell are present in this field. (48-hour culture in chicken plasma and blood-serum from a sarcoma-bearing rat; acid alcohol; iron-hæmatoxylin and Sudan III.)

FIG. 6.—Fibroblast-like cell as shown by dark-ground illumination. Note the numerous mitochondria and fat-globules. (4-day culture grown in chicken plasma and homologous rat serum.)

FIG. 7.—Binucleate fibroblast-like cell as shown by dark-ground illumination from a 48-hour culture grown in normal rat serum. (This cell degenerated two hours after drawing.)

FIG. 8.—Wandering cell as illuminated by the dark-ground method. (24-hour culture in chicken plasma and serum from a tumour-bearing rat.)

FIG. 9.—Series of drawings of a wandering cell examined by dark-ground illumination at body temperature. The drawings were made at intervals of one minute, and show the rapid changes in form characteristic of these cells. (20-hour culture in chicken plasma and serum from a tumour-bearing rat.)

FIG. 10.—Normal mesenchyme cell from a rat embryo as shown by dark-ground illumination.

FIG. 11.—Series of drawings showing mitosis in a fibroblast-like sarcoma-cell. The drawings were made from the living cell at body temperature by direct illumination. (3-day culture in chicken plasma and serum from a tumour-bearing rat.)

FIG. 12.—Series of drawings made from a living cell by direct illumination, showing the formation of a binucleate element by means of incomplete division. (5-day culture in chicken plasma and serum from a tumour-bearing rat.)

FIG. 13.—Multinucleate cell of the fibroblastic type. Note the very varied sizes of the nuclei. (72-hour culture in blood-serum from a sarcoma-bearing rat; Zenker; hæmatoxylin and eosin.)

FIG. 14.—"Epithelioid" cell in a culture of Jensen's rat sarcoma. (24-hour culture in blood serum from a tumour-bearing rat; 2% osmic acid; iron-hæmatoxylin and acid fuchsin.)

FIG. 15.—*a*. Normal fibroblast-like cell from a 72-hour culture in blood-serum from a sarcoma-bearing rat (2% osmic acid; methyl green and acid fuchsin.) *b*. Hypertrophied fibroblast-like cell drawn to the same scale. Note the numerous apparently normal mitochondria. (11-day culture in blood-serum from a sarcoma-bearing rat; 2% osmic acid followed by Zenker; hæmatoxylin.)

FIG. 16.—Series of drawings made from a living cell at body temperature by dark-ground illumination showing the degeneration of a wandering cell. (3-day culture in chicken plasma and malignant rat serum.)

FIG. 17.—Fibroblast-like cells and wandering cells showing fat-globules. (72-hour culture in chicken plasma and blood-serum from a sarcoma-bearing rat; acid alcohol; iron-hæmatoxylin and Sudan III.)

FIG. 18.—Wandering cell showing mitochondria, cell-granules and fat-vacuoles (fat dissolved out). This cell was completely isolated from its neighbours. (48-hour culture in blood-serum from a sarcoma-bearing rat; 2% osmic acid; acid fuchsin.)

FIG. 19.—Fibroblast-like cells showing mitochondria. (72-hour culture in blood-serum from a tumour-bearing rat; 2% osmic acid; methyl green and acid fuchsin.)

FIG. 20.—Wandering cell and fibroblast-like cell in a culture to which grains of carmine had been added. In the wandering cell the cell-granules have been stained *intra vitam* by the carmine; an ingested carmine grain is also present. (7-day culture in chicken plasma and blood-serum from a sarcoma-bearing rat; acid alcohol; iron-hæmatoxylin.)

FIG. 21.—Fibroblast-like cell in an old culture showing enlarged centrosphere. (7-day culture in serum from a sarcoma-bearing rat; Zenker; Grübler's methylene blue-eosin.)

FIG. 22.—"Epithelioid" cell showing brilliantly stained cell-granules. (48-hour culture in serum from a sarcoma-bearing rat; Flemming's solution without acetic acid followed by Müller's solution; acid fuchsin and alcoholic methyl green.)

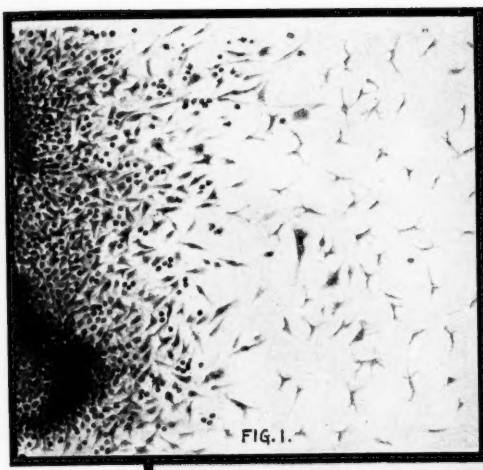

FIG. 1.

FIG. 2.

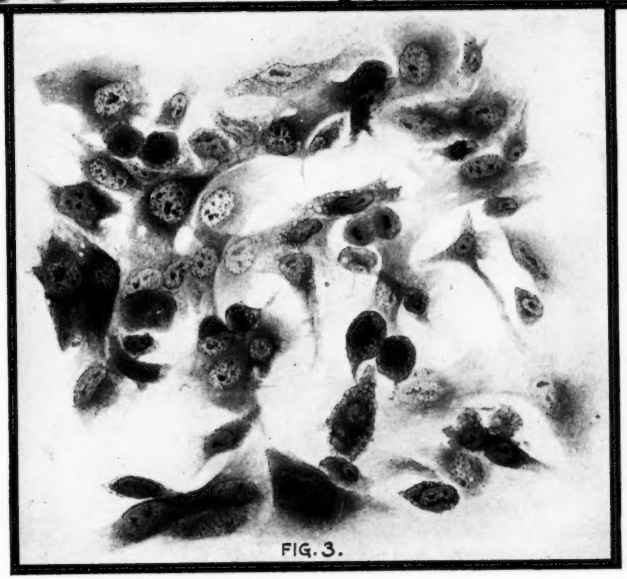

FIG. 3.

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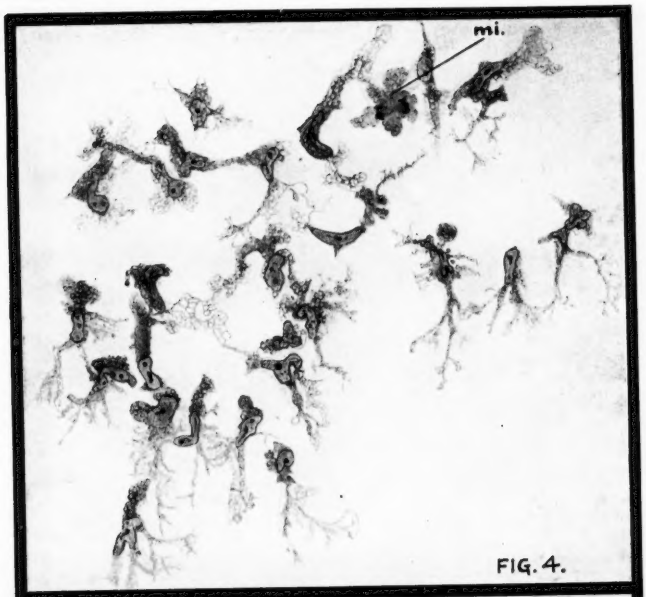

FIG. 4.

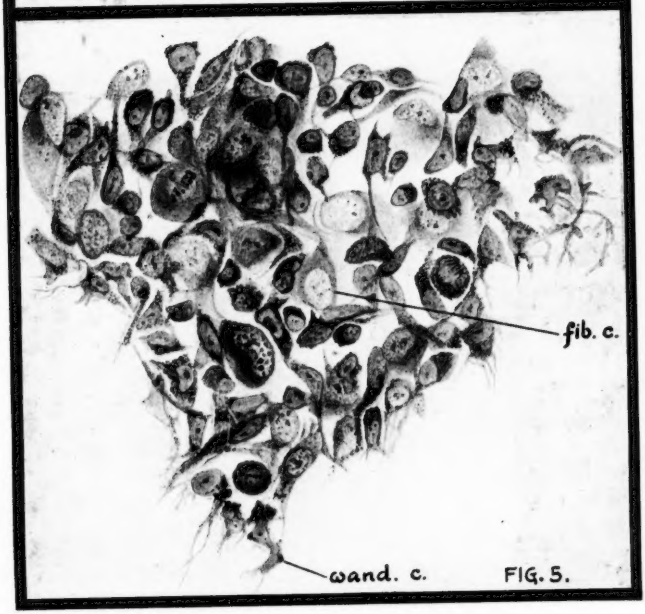

FIG. 5.

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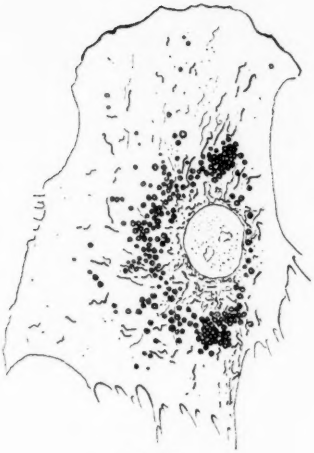

FIG. 6.

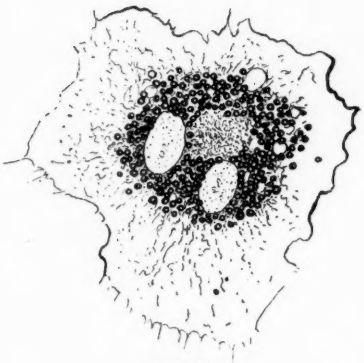

FIG. 7.

FIG. 8.

FIG. 9.

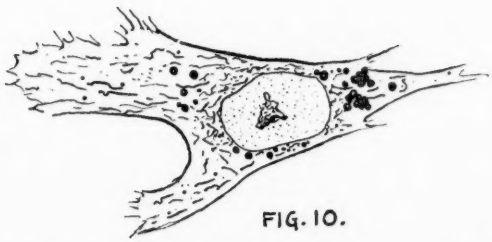

FIG. 10.

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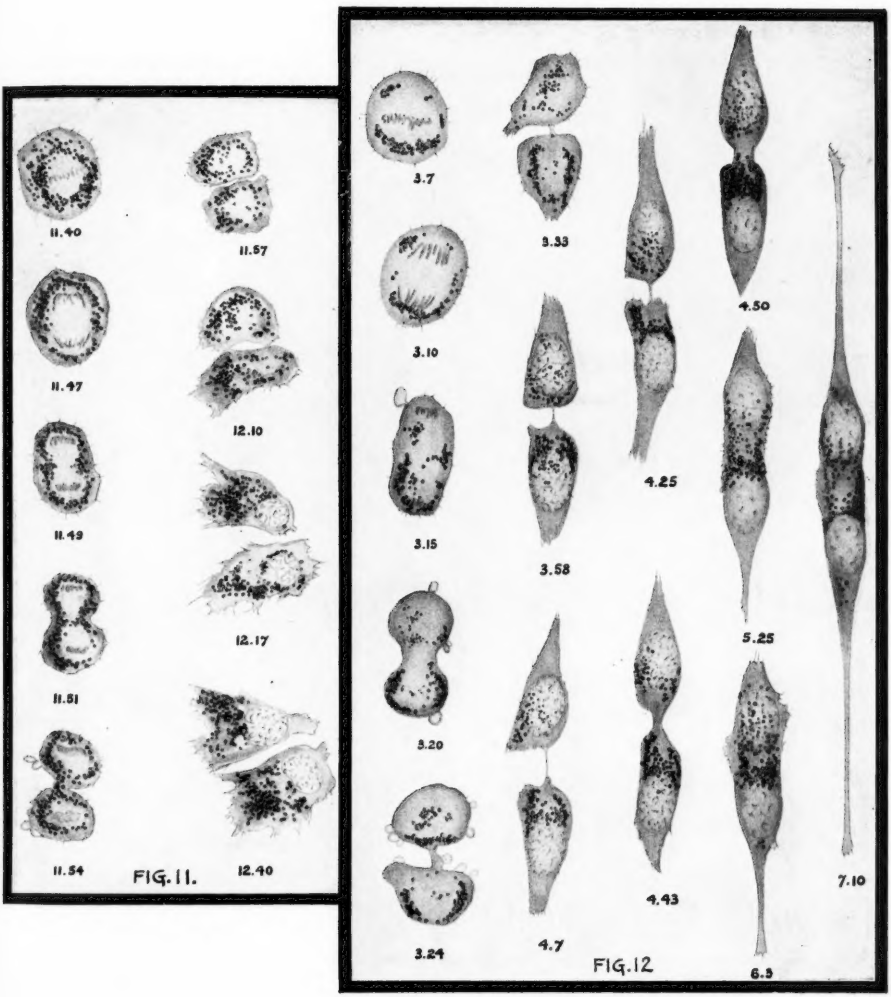
11.40
11.57
11.47
12.10
11.49
12.17
11.51
11.54
12.40
3.7
3.33
3.10
4.50
4.25
3.15
3.58
5.25
5.20
4.43
7.10
3.24
4.7
6.3

FIG. 11.

FIG. 12

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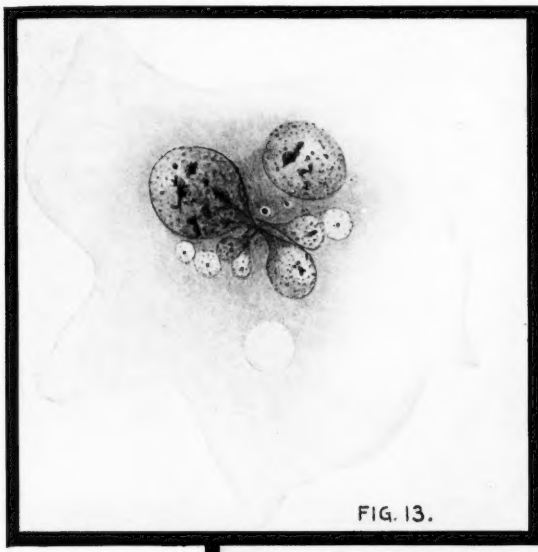

FIG. 13.

FIG. 14.

FIG. 15.

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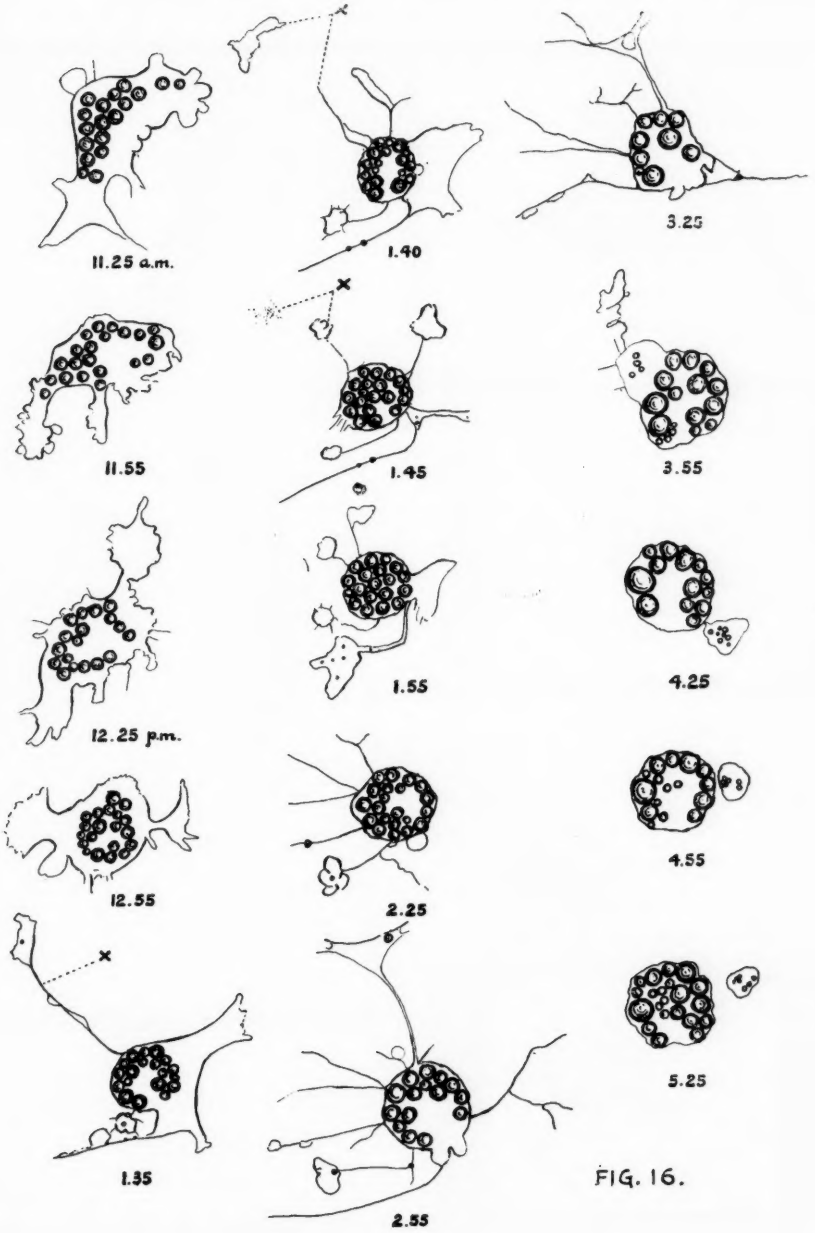

FIG. 16.

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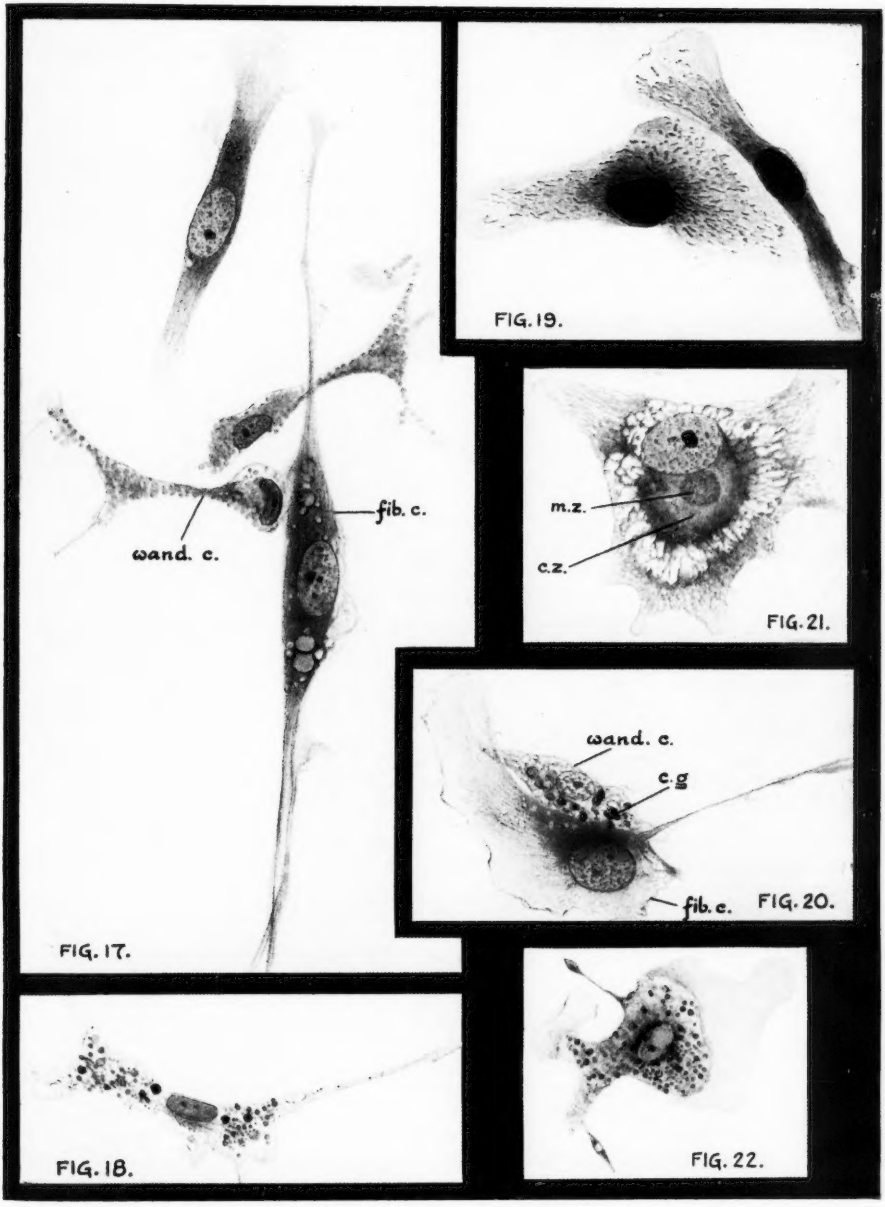
wand. c.
fib. c.
FIG. 17.
FIG. 19.
m.z.
c.z.
FIG. 21.
wand. c.
c.g
fib. c.
FIG. 20.
FIG. 18.
FIG. 22.

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BLOOD-PHOSPHORUS IN HEALTH AND DISEASE.—II. CHANGES IN THE PHOSPHORUS COMPOUNDS OF THE BLOOD IN RENAL DISEASE.*

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GREENWALD in 1915 showed that in many cases of nephritis, not only was the acid-soluble phosphorus of serum markedly increased, but also the lipin phosphorus frequently above normal. Since that year considerable attention has been directed towards the level of the inorganic phosphorus, and, to a less extent, of the lipin phosphorus of the blood, the plasma or the serum in renal disease. Greenwald's findings have been extended in various directions (Marriott and Howland, 1916; Bloor, 1917; Denis and Minot, 1920; Denis and Hobson, 1923; de Wesselow, 1923; Daniels, 1925; Oser and Karr, 1925), and the early work which established the fairly close correspondence existing between the retention of nitrogen and of phosphorus has been confirmed.

When it is remembered that in the normal individual the inorganic phosphate of blood is only one-twelfth of the total phosphorus, it is surprising that the other phosphorus compounds, particularly those represented in the fraction we call the "ester phosphorus," have been so little studied. One of the objects of the present investigation has been to ascertain whether in nephritis the phosphorus compounds of the blood, other than inorganic phosphate, suffer changes corresponding with those undergone by the inorganic phosphate, and, if so, whether any prognostic value can be attached to such changes.

We have examined thirty-nine cases of nephritis and other renal disorders of varying severity, carrying out fairly complete phosphorus analyses in each case. A few examples of "surgical" lesions of the kidney, such as hydronephrosis and renal calculus, have been included. Whenever kidney function has been found to be seriously impaired, the changes observed in the blood have been, on the whole, akin to those which occur in true Bright's disease.

In all investigations of this nature the initial stumbling-block, always difficult to overcome, is the clinical classification of the types of nephritis to which the individual cases belong. In this paper a simple separation into acute, subacute and chronic forms has been adopted. By *acute* nephritis is meant a first attack from which apparent recovery ensues within a period of weeks. By *subacute* nephritis is meant a primary attack of more prolonged duration in which the disease has lasted for a period of months. By *chronic* nephritis is meant a prolonged attack in which the course of the disease is

* Some of the results described in this paper were submitted by one of us (F. B. B.) in a thesis accepted in partial fulfilment of the requirements for the degree of M.D. (London).

TABLE I.—*Nephritis.*

No.	Case.	Inorganic P.	Total acid soluble P.	Ester P.	Hydrolysable ester P.	Non-hydrolysable ester P.	Lipin P.	Grand total.	Hæmatocrit.	Organic phosphorus index.	Lipin P.		Blood-urea %.
											Plasma.	Corpuscles (calc.).	
	Average normal .	3·1	25·8	22·7	6·7	16·0	12·0	37·8	43·0	53	7·1	18·0	·03
1	Acute nephritis .	4·2	26·4	22·2	7·5	14·7	10·5	36·9	37·5	59	6·7	16·9	·19
2	„ „ .	3·3	27·6	24·3	6·7	17·6	10·3	37·9	43·0	56	...	...	·05
3	„ „ .	3·4	28·8	25·4	5·9	19·5	10·6	39·4	42·0	60	...	...	·04
4	„ „ .	4·0	24·9	20·9	7·2	13·7	...	...	41·5	53	...	...	·03
5	„ „ .	4·4	24·9	20·5	6·6	13·9	8·7	33·6	32·0	60	...	...	·09
6	„ „ .	4·5	25·2	20·7	8·0	12·7	11·5	36·7	35·0	59	8·5	17·4	·05
7	„ „ .	3·6	32·0	28·4	7·7	20·7	11·1	43·1	45·0	63	...	...	...
8	„ „ .	4·5	24·9	20·4	6·9	13·5	10·3	35·2	37·5	54	6·7	16·3	·05
9	Subacute nephritis	6·0	28·8	22·8	5·0	17·8	12·5	41·3	34·0	67	...	...	·05
10	„ „ .	6·3	27·6	21·3	9·4	11·9	15·6	43·2	35·5	56	14·0	18·6	·09
+11	„ „ .	5·0	22·1	17·1	4·8	12·3	9·3	31·4	25·5	67	...	...	·20
12	„ „ .	4·5	24·7	20·2	9·2	11·0	15·0	39·7	38·5	52	13·0	18·1	04
13	„ „ .	4·3	17·8	13·5	6·1	7·4	12·7	30·5	24·0	56	...	...	·17
14	„ „ .	4·1	29·2	25·1	7·2	17·9	14·0	43·2	41·5	60	...	...	·07
+15	Chronic nephritis .	16·0	27·7	11·7	9·6	2·1	11·4	39·1	16·3	72	9·9	19·0	·51
+16	„ „ .	12·3	22·0	9·6	6·3	3·3	10·5	32·5	17·5	55	...	...	·20
+17	„ „ .	11·7	21·2	9·5	5·3	4·2	9·6	30·8	16·0	59	...	...	·19
18	„ „ .	7·7	27·2	19·5	9·3	10·2	12·2	39·4	34·0	57	...	...	·16
+19	„ „ .	7·5	23·1	15·6	6·4	9·2	12·5	35·6	20·5	76	10·7	19·5	·40
+20	„ „ .	10·3	29·3	19·0	9·4	9·6	12·5	41·8	27·0	70	9·9	19·2	·30
+21	„ „ .	8·2	33·4	25·2	11·1	14·1	10·8	44·2	38·0	67	7·2	16·5	·24
+21a	„ „ .	8·1	25·2	17·1	7·7	9·4	11·0	36·2	19·5	88	...	...	·22
+22	„ „ .	5·9	19·2	13·3	6·0	7·3	10·9	30·1	20·5	65	...	...	·17
23	„ „ .	3·8	25·8	22·0	8·1	13·9	12·0	37·8	41·0	53	...	...	·09
+24	„ „ .	3·3	30·0	26·7	9·3	17·4	12·4	42·4	31·0	86	...	...	·13
25	„ „ .	3·1	24·3	21·2	7·5	14·7	11·9	36·3	39·0	54	8·5	17·3	...
26	„ „ .	3·9	26·7	22·8	7·0	15·8	14·6	41·3	38·0	60	12·9	17·5	·10
27	„ „ .	2·8	24·5	21·7	5·0	16·7	12·6	37·1	39·0	55	8·9	18·5	·03
28	Hyperpiesia . .	3·5	30·5	27·0	8·7	18·3	11·4	41·9	50·0	53	...	...	·03
	Surgical Lesions, etc.												
+29	Renal calculi . .	20·6	37·6	17·0	12·4	4·6	12·0	49·6	24·5	69	10·6	16·4	·29
+30	Hydronephrosis .	13·8	25·6	11·8	7·1	4·7	...	...	20·0	59	7·6	...	...
+31	„ .	8·7	19·3	10·6	5·6	5·0	8·5	27·8	21·5	49	6·1	17·2	·39
+32	Renal tuberculosis .	8·4	31·0	22·6	...	...	11·5	42·5	38·0	59	...	...	·16
+33	Renal calculi . .	11·1	31·6	20·5	9·5	11·5	9·0	40·6	32·0	64	4·5	18·5	·31
34	Pyelonephritis .	3·9	30·3	26·4	6·3	20·1	12·9	43·2	45·5	58	...	...	·07
35	Acute gout . .	4·7	26·6	21·9	4·1	17·8	9·4	36·0	27·0	81	6·8	16·5	·10
36	Pyæmic nephritis .	*13·8	48·4	34·6	7·9	26·7	11·2	59·6	33·0	105	...	...	·30
37	Pyelonephritis .	4·6	25·1	20·5	6·7	13·8	12·0	37·1	32·5	63	8·8	18·7	·07
38	„ .	3·4	28·9	25·5	7·7	17·8	14·0	42·9	34·0	75	...	...	·10

* Leucocyte volume = 2·3% of whole blood (normal leucocyte volume = 0·7% of whole blood).

\+ Died within seven weeks of the date of the blood analysis.

TABLE II.—*Averages of Determinations in three Clinical Groups.*

Group.	P in mgm. P per 100 c.c. blood.							% corpuscles (hæmatocrit).	Organic P index.	Hydrolysable P index.	Non-hydrolysable P index.	Blood-urea %.
	Inorganic.	Total acid soluble.	Ester.	Hydrolysable.	Non-hydrolysable.	Lipin.	Total.					
Normal . . .	3·1	25·8	22·7	6·7	16·0	12·0	37·8	43·0	53	15·7	37·2	0·03
Acute nephritis .	4·0	26·8	22·8	7·1	15·7	10·4	37·2	39·2	58	18·0	40·0	0·08
Subacute nephritis .	5·0	25·0	20·0	7·0	13·0	13·8	38·8	33·2	60	21·1	39·0	0·10
Chronic nephritis . (all cases)	7·5	25·7	18·2	7·7	10·5	11·8	37·5	28·3	65	27·2	37·1	0·21
Chronic nephritis . (moribund cases)	9·9	25·4	15·5	7·9	7·6	11·3	36·7	23·7	65	33·0	32·0	0·24

Explanation of Headings in Tables I and II.

Inorganic P = P present in form of salts of ortho-phosphoric acid. *Total acid soluble P* = P present in all phosphorus compounds which are not precipitated from blood by coagulation of proteins with trichloro-acetic acid. *Ester P* = total acid-soluble P less inorganic P. *Hydrolysable P* = portion of ester P which is hydrolysed to inorganic phosphate, under certain conditions, by phosphatases. *Non-hydrolysable P* = portion of ester P not so hydrolysed. *Lipin P* = P of whole blood or plasma which is soluble in alcohol-ether. *Total P* = total P of whole blood as determined either by direct combustion or by adding lipin P to total acid-soluble P. *Organic P index* = amount of ester P in mgm. per 100 c.c. of red cells.

measured in years, or a case in which examination of the patient reveals by the presence of high blood-pressure, cardio-vascular hypertrophy, anæmia, fixation of specific gravity of the urine, high blood-urea, etc., that the degree of destruction of kidney tissue has already progressed to an advanced stage. Patients with ischæmic degeneration resulting from the vascular changes following prolonged "primary high blood-pressure" (*i. e.* cases of hyperpiesia with secondary renal degeneration) are not included by us in any of these three types.

RESULTS.

The blood analysis was conducted on lines which have recently been described (Kay and Byrom, 1927), with slight modifications in certain cases where the hæmatocrit reading was low. The results are collected in Table I.

The individual figures in each class show very considerable variation in every kind of *organic* phosphorus in the blood, as well as in the inorganic phosphorus. But if averages are taken of cases in each of the three clinical groupings, together with the better-known changes in inorganic phosphorus, certain progressive changes in the corpuscular content of organic phosphorus are observed (Table II).

COMMENTARY.

(a) *Inorganic Phosphate.*

Our findings are in accordance with those of other workers. There is a variable, occasionally an extreme, increase in the inorganic phosphate content of whole blood or of plasma. The degree of retention of inorganic phosphate

is roughly proportional to the severity of the disease as judged clinically, or as assessed by the level of the blood-urea and by the usual renal efficiency tests. Marked phosphorus retention appears, however, to begin later than urea retention, and in our experience is a very serious prognostic sign (confirming de Wesselow, 1924). Thus in every instance in which we have recorded an inorganic phosphate of more than 8 mgm. P per 100 c.c., the patient died, usually within a week or two. The converse does not always hold—a premortal increase of inorganic phosphorus is not always observed. Case 24 is a good example of such an exception. Clinically the case was a typical one of chronic nephritis, although the blood inorganic phosphate had not risen above 3·3 mgm. P % on the two occasions on which it was estimated. The patient was, on the latter occasion, in coma, with a blood-urea of 0·13 %. He died five days afterwards, and the post-mortem examination confirmed the diagnosis of chronic nephritis.

(b) *Total Acid-soluble Phosphorus* (= inorganic P + ester P).

If there was a marked increase in the inorganic phosphate (as in the later stages of nephritis) this group of phosphorus compounds, comprising, as it does, the inorganic phosphorus of whole blood and the ester phosphorus of the corpuscles, might be expected to rise also. Nevertheless, there is usually no such rise, as the high inorganic phosphorous is almost always associated with profound anæmia. As 98–99 % of the ester phosphorus is confined to the corpuscles, there is therefore a paucity of ester phosphorus per 100 c.c. whole blood. In fact the lowest figures for total acid-soluble are usually recorded in those cases in which the increase in inorganic phosphorus (and in the anæmia) is very marked. The secondary anæmia in the terminal stages of chronic nephritis, particularly in those dying in uræmia, is one of the most severe secondary anæmias encountered. The degree of anæmia is not far short of that found in severe cases of primary blood disease, and appears to be one of the best clinical indications of the state of any given case.

(c) *Ester Phosphorus.*

The ester phosphorus in nephritis, as in normal blood, is almost entirely confined to the corpuscles. (The *plasma* ester phosphorus in 10 normals we have found to average only 0·3 mgm. per 100 c.c. The average value for 16 nephritics is 0·5 mgm.) For the ester phosphorus in the blood in nephritics the following rough relationships hold: (*a*) ester P $\propto$ percentage red cells; (*b*) ester P $\propto$ 1/inorganic P; (*c*) ester P $\propto$ 1/blood-urea.

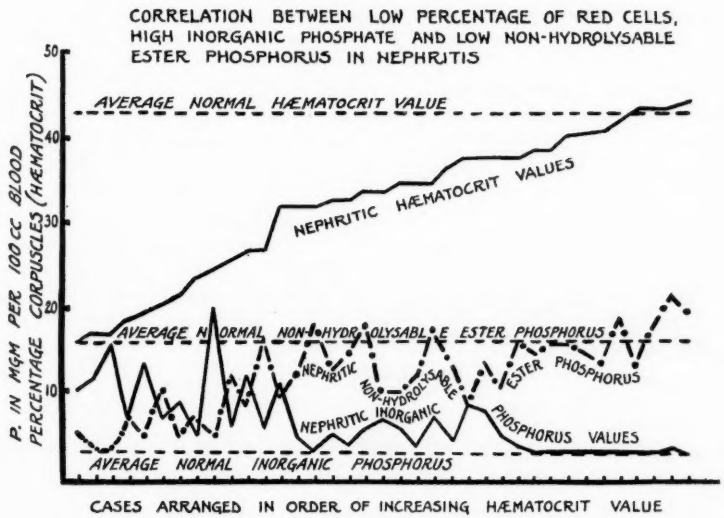
(d) *The Enzyme-hydrolysable Phosphorus.*

Our results indicate that there is a marked fall in the ester phosphorus in whole blood in advanced, chronic nephritis, which, apparently, is due entirely to the secondary anæmia. It remains to be decided whether the normal relationship of the two fractions, *i. e.* that portion which is hydrolysable under certain conditions by phosphatase, and that portion which is not hydrolysable, is altered in this disease. In health the ratio between the hydrolysable and the non-hydrolysable is 1 : 2·4. A scrutiny of Table I shows that in addition

to the fall in the total ester phosphorus, there is also a change in the *proportion* of the two fractions, the hydrolysable remaining normal or above normal, while the non-hydrolysable phosphorus is much reduced. In the most severe cases (*cf.* cases 15, 16, 17) the normal proportion of hydrolysable : non-hydrolysable of 1 : 2·4 may even be reversed.

In Table II the findings have been averaged for each of the three main clinical groups and compared with normal average figures. This process of averaging is perhaps a dangerous one to employ; not only do the individual figures vary widely, but the patients concerned are in different stages of the disease, and present widely differing degrees of disturbed renal function. The average figures would seem, nevertheless, to indicate general tendencies, which

FIG. 1.


CORRELATION BETWEEN LOW PERCENTAGE OF RED CELLS, HIGH INORGANIC PHOSPHATE AND LOW NON-HYDROLYSABLE ESTER PHOSPHORUS IN NEPHRITIS
P. IN MGM PER 100 CC BLOOD
PERCENTAGE CORPUSCLES (HÆMATOCRIT)
50
40
30
20
10
AVERAGE NORMAL HÆMATOCRIT VALUE
NEPHRITIC HÆMATOCRIT VALUES
AVERAGE NORMAL NON-HYDROLYSABLE ESTER PHOSPHORUS
NEPHRITIC NON-HYDROLYSABLE ESTER PHOSPHORUS
NEPHRITIC INORGANIC PHOSPHORUS VALUES
AVERAGE NORMAL INORGANIC PHOSPHORUS
CASES ARRANGED IN ORDER OF INCREASING HÆMATOCRIT VALUE

are borne out by further study of the main table. It is clear that associated with a small rise in the total amount of ester phosphorus in 100 c.c. corpuscles there is a more marked change in its nature, the hydrolysable fraction of the ester phosphorus increasing as the disease becomes chronic. We have shown previously that the ester phosphorus compounds probably form an integral part of the cellular furniture of the erythrocyte. The present finding indicates therefore that the chemical composition of the red cell—one of the very few human cells it is possible to investigate without delay in the laboratory—changes qualitatively in nephritis.

Moreover, this qualitative change varies with the severity of the disease. One fact of importance does not emerge clearly from the table of averages. This is the drop in the non-hydrolysable phosphorus in *severe* chronic nephritis, which in certain cases (*vide* 15, 16, 17) is almost as striking as the rise in the inorganic phosphate of the blood. Fig. 1 shows the correlation between a low

percentage of red cells, a high inorganic phosphate, and a low non-hydrolysable ester phosphorus, and also reveals that the fall, in individual cases, in the non-hydrolysable ester phosphorus of the blood is almost always accompanied by a corresponding rise in the inorganic phosphorus. The dips in the former curve correspond regularly with the peaks in the latter.

It was shown some time ago by one of us (Kay, 1924) that ammonium chloride acidosis was accompanied by a diminution, small, but apparently quite definite, in the non-hydrolysable portion of the ester phosphorus of the corpuscle, which diminution was greatest at the peak of the acidosis. If the amount of inorganic phosphorus of the blood in nephritis can be taken as an indication of the degree of acidosis, as suggested by Marriott and Howland (1916), then here, again, severe acidosis is associated with a diminution in the corpuscle content of non-hydrolysable phosphoric ester. One is tempted to take the view that in the endeavour to adjust itself to severe acidotic conditions, especially the final stages of an acidosis of long standing, the red cell is compelled to jettison a portion of its cargo of phosphorus, and the non-hydrolysable ester phosphorus can most easily be spared. In prolonged acidosis the corpuscular content of base may be so far depleted that, to preserve the corpuscle in functional activity, acidic radicles such as those of the phosphoric esters must also be extruded. (Such an effect may not be confined to the cells of the blood.)

The fall in the non-hydrolysable ester phosphorus becomes really marked, however, only in the last stages of the disease, a non-hydrolysable ester phosphorus of 10 mgm. P % or less indicating, in our experience, an early fatal termination. In only one of eleven cases of renal disease with a non-hydrolysable ester phosphorus of less than 10 mgm. % has the patient survived more than six weeks.

(e) *The Phosphorus Index in Nephritis.*

We have defined* (1927) the phosphorus index of blood as the amount of ester phosphorus in milligrammes present in 100 c.c. of red cells. This has been shown to be remarkably constant, in health, at 53 mgm. In calculating the value for normal individuals we have wittingly omitted two small corrections, one for the amount of ester phosphorus present in the leucocytes, and the other for the ester phosphorus content of the plasma. While this omission may be permitted for normal blood, it is not admissible in cases where the relative red cell content of the blood is low, or there is a leucocytosis, or both conditions are present together.

On the basis of ascertained figures for (1) the average phosphorus content of the leucocyte, (2) the proportion of leucocytes, (3) the ester phosphorus of the *plasma* in nephritic blood, we have calculated that the maximum correction for leucocyte phosphorus to be applied to the apparent phosphorus index is a diminution of 4·5 units (case 22). For plasma ester phosphorus the maximum correction is a subtraction of 2·7 units (case 17). It may be concluded that on the whole, in nephritis, an index of above 60 represents a real increase in

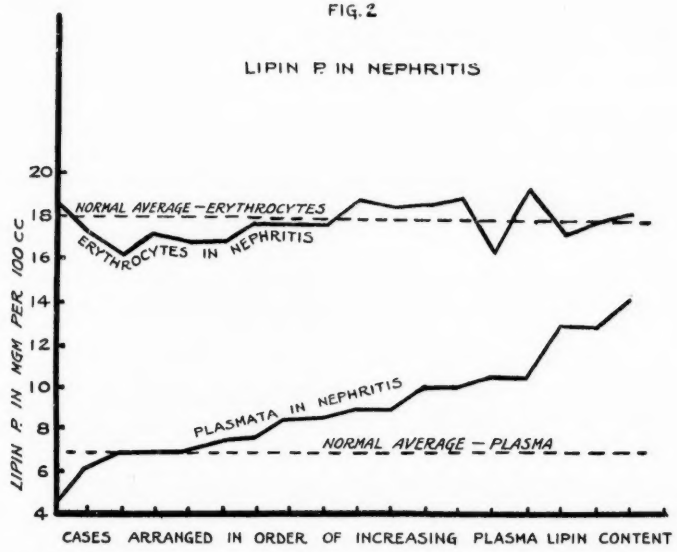
* *Correction.*—By error the word "ester" was omitted before the word "phosphorus" in the third line of paragraph *F*, p. 249, of our 1927 paper.

the red cell phosphorus. Of those we have examined, 13% of the acute, 33% of the subacute, and 50% of the chronic cases show such an increase.

(f) *Lipin Phosphorus.*

We have found that the ether-soluble phosphorus of whole blood is, on the average, slightly diminished in acute, slightly increased in subacute, and almost normal in chronic nephritis. Bloor (1918), however, has shown that in normal blood there is considerably more lipin phosphorus in the corpuscles than in the plasma, and this we have found to be the case in the blood in nephritis.

FIG. 2



In about half the cases detailed in Table I we have determined both plasma and whole blood lipin phosphorus (corpuscle lipin by calculation), and have found that in the majority of these cases the plasma lipin P is above normal, while the corpuscular lipin P, taking the possible experimental errors into account, remains constant and very nearly normal (see Fig. 2). In nine cases of subacute and chronic nephritis the whole blood lipin phosphorus varies from 10·8 to 15·6 mgm. per 100 c.c., with an average value of 13·1 mgm. (average normal lipin P = 12·0 mgm.), whilst the plasma lipin P varies from 7·2 to 14·0 mgm. per 100 c.c., with an average value of 10·6 mgm. (average normal lipin P = 7·1 mgm.). Daniels (1925) found a substantial increase in plasma "lecithin" in five out of seven cases of chronic parenchymatous nephritis. Oser and Karr (1925) also found increases in plasma "lecithin" in several cases of uræmia.

The original observation of Greenwald (1915) that the plasma lipin phosphorus is often increased in nephritis is therefore abundantly confirmed.

THE VALUE OF BLOOD P DETERMINATIONS IN THE STUDY OF PATIENTS WITH RENAL DISEASE.

The most important alterations in the phosphorus compounds of the blood in severe renal disease are an increase in the inorganic phosphate of whole blood (or plasma) and a diminution in the non-hydrolysable fraction of the ester phosphorus of the corpuscles. A blood which has a high inorganic phosphorus (5 mgm. % or above), a high blood-urea (0·06% or above), with a low hæmatocrit value (35% corpuscles or below), and in which the corpuscles sink rapidly, is characteristic of advanced chronic nephritis. In our own experience an inorganic phosphorus of 7 mgm. % or above, and particularly a low non-hydrolysable ester phosphorus (10 mgm. % or under), are of the gravest import, and point to death within a period of weeks.

SUMMARY.

The distribution of four types of phosphorus compounds in the blood of 39 cases of renal disease has been determined. The increase in inorganic phosphorus (and in lipin phosphorus in subacute nephritis) observed by previous workers has been confirmed. In addition to these changes in the inorganic and lipin fractions, definite changes have been shown to occur in the organic acid-soluble ("ester") phosphorus of the red cells. This fraction is altered both in nature and in quantity in advanced nephritis, thus demonstrating a deep-seated change in the chemical composition of the erythrocyte itself.

We are grateful to Prof. Arthur Ellis for suggestions and helpful criticism.

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THE NATURE OF THE ACTION OF ULTRA-VIOLET LIGHT ON MICRO-ORGANISMS.

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The purpose of this communication is to describe an investigation of the action of ultra-violet light in destroying micro-organisms. It is hoped to be able to show (1) that ultra-violet light is capable under ordinary conditions of producing hydrogen peroxide; (2) that the destruction of micro-organisms by ultra-violet light is due and is directly proportional to the production of this substance; (3) that the relative susceptibility of different micro-organisms to ultra-violet light is of the same order as their relative susceptibility to hydrogen peroxide as tested *in vitro*. Further, it is proposed to draw some conclusions as to the mode of action of the fluorescent antiseptics.

Evidence by Chemical Tests of the Production of Hydrogen Peroxide by Ultra-Violet Light.

There is really no reliable specific test for hydrogen peroxide in minute quantities. The starch-iodide and titanium sulphate colour reactions can both be obtained, but are both non-specific and unreliable. The following results were obtained using a water-cooled Kromayer (Quartzlampe Wechselsform), which will hereafter be designated as "the lamp":

Starch-iodide solution in watch-glass—blue colour produced in 4 minutes.

Titanium sulphate solution in watch-glass—yellow colour produced in 20 minutes.

In each case the lamp was 4 in. from the solution.

Similar results were obtained when ordinary agar plates, dried in the incubator for 15 minutes, were irradiated and test solution poured on to the surface of the medium. The colour change in the titanium sulphate solution was difficult to gauge by direct examination, but on pouring off and observing in a comparator with control, definite alterations could always be made out. It was found possible to incorporate starch-iodide solution in the agar medium. On irradiating such a preparation it became evident that blue formation was a surface phenomenon, and did not extend more than a very short distance into the depth of the medium—only by irradiating with the medium very close to the lamp, and for a long time, was it at all possible to bring about formation below the surface.

It is now known (McLeod and Gordon, 1922) that the green coloration produced on a heated blood agar plate by the early growth of an organism such as the pneumococcus is due to hydrogen peroxide formation. If, therefore,

ultra-violet light produces hydrogen peroxide, it should be possible on irradiating such a plate to obtain a similar green colour.

A chocolate plate (heated blood agar) was covered by an opaque mask, leaving, however, a small T-shaped area bare. The whole was now irradiated at a distance of 3 in. for forty minutes. On removing the mask there was found a strikingly clear-cut green T, indistinguishable in colour from that produced by the pneumococcus.

It is clear that the above findings, although very suggestive, do not provide definite proof of hydrogen peroxide formation. The writer has no wish to exaggerate their importance, and would only have them recognized in the light of "additional evidence" to be taken into consideration with what is to follow.

The Effect of Irradiation of Ordinary Agar upon the Growth of subsequently Inoculated Organisms.

In this experiment the inoculum consisted of an emulsion of a 24 hours' agar culture of the organism standardized by the opacity method. Inoculation was performed with the platinum loop. Each inoculation was made 5 minutes after exposure, and the plate at once deposited in the incubator. A reading was taken after 18 hours' incubation at 37° C. In all these experiments irradiation was performed directly on to the medium without the intervention of glass.

Table I embodies the average of twelve experiments. All plates were exposed uncovered at a distance of 12 in. The lamp was at the same intensity throughout the experiments. The plates were inoculated after exposure. The table shows degree of inhibition.

TABLE I.

Time.	*V. choleræ.*	*B. diphtheriæ.*	Staphylococcus.	*B. Hofmanni.*	*B. typhosus.*
5 mins.	No	No	No	No	No
10 ,,	Slight	,,	,,	,,	,,
20 ,,	Marked	Slight	,,	,,	,,
40 ,,	Complete	Marked	Slight	Very slight	,,

It is obvious from Table I (*a*) that irradiation of ordinary agar media has a profound influence on the growth of organisms subsequently inoculated thereupon; (*b*) that different organisms are inhibited to different degrees, and that it is possible to arrange a descending order of susceptibility with *V. choleræ*, the most susceptible of the organisms tested, at one end, and *B. typhosus*, the least susceptible, at the other. It is evident that inhibition is due to some substance formed in the media during irradiation.

The Effect of Irradiation of Ordinary Agar upon the Growth of previously Inoculated Organisms.

In Table II the constants are the same as in Table I, the sole difference being that the plate is exposed with the organisms already inoculated. The average of eight experiments is given:

TABLE II.

Time.	*V. choleræ.*	*B. diphtheriæ.*	Staphylococcus.	*B. Hofmanni.*	*B. typhosus.*
15 secs.	No	No	No	No	No
30 ,,	Slight	,,	,,	,,	,,
1 min.	Marked	Slight	,,	,,	,,
3 mins.	Complete	Marked	Slight	,,	,,
5 ,,	,,	,,	Marked	Slight	Very slight

On inspection it is obvious that the results in Tables I and II are similar, with the exception that the destructive action is much more intense and rapid in the second series of experiments. The relative order of susceptibility is the same in both. We may therefore conclude that the substance which was responsible for the inhibition is the same in both series of experiments.

The Nature of the Inhibitory Substance.

We have seen that by chemical tests a certain amount of evidence can be obtained indicating hydrogen peroxide formation by ultra-violet light. Is hydrogen peroxide the inhibitory substance?

Ordinary agar contains no catalase; if, however, we use a medium rich in catalase the inhibitory action of an irradiated plate should be reduced to nothing, or at the least considerably diminished, if hydrogen peroxide is, as we have suggested, the sole cause of inhibition. Blood agar, which always contains much catalase, provided the heating has not been too prolonged, is a good medium for the test. Table III gives the results of such an experiment.

The blood agar was irradiated previous to inoculation. The constants are as in Tables I and II.

TABLE III.

Time.	*V. choleræ.*	*B. diphtheriæ.*	Staphylococcus.	*B. Hofmanni.*	Typhoid.
5 mins.	No	No	No	No	No
10 ,,	,,	,,	,,	,,	,,
20 ,,	Very slight	,,	,,	,,	,,
40 ,,	Slight	Doubtful	,,	,,	,,

The above findings are striking and conclusive.

Still further evidence that the inhibitory substance is hydrogen peroxide can be obtained by comparing the relative susceptibility of the different micro-organisms to hydrogen peroxide *in vitro* with that to ultra-violet rays. McLeod and Gordon (1923) give a list of the relative susceptibility of various organisms to hydrogen peroxide. The absolute figures do not concern us here, but their order of relative susceptibility is important. They give *V. choleræ* most susceptible, then *B. diphtheriæ*, then *Staphylococcus aureus*, next *B. Hofmanni*, and least susceptible of all, *B. typhosus*. This order of relative susceptibility

is identical with that found by the writer for the susceptibility of these organisms to ultra-violet light.

It is therefore maintained that ultra-violet rays exert an influence on micro-organisms in virtue of the hydrogen peroxide they produce, and that the intense action of the direct rays is due to a production of hydrogen peroxide within the organisms themselves. In no way is this view contrary to that of Clark (1922), who, in an extensive review of the literature, expresses the opinion that the lethal action of ultra-violet light is consequent upon changes in the nature and condition of the proteins of the organisms concerned. That such changes occur is not disputed (Schanz, 1918; Chalupecky, 1918; Bovie and Klein, 1913), but it is the writer's contention that the changes which determine the death of the organism in these experiments are mainly a result of hydrogen peroxide formation.

It will have been observed that so far no data have been submitted illustrating the action of ultra-violet light on the strict anaerobes. Here the experimental difficulties are considerable, and the number of experiments is as yet insufficient to warrant publication.

Photodynamic Sensitization.

No consideration of ultra-violet light will be complete without reference to photodynamic sensitizers. Ordinary visible light has only a very mild effect upon micro-organisms. It is, however, possible to sensitize them just as one sensitizes a photographic plate, and produce an abnormal condition in which visible light is as active as ultra-violet light. This phenomenon of photo-sensitization was first investigated in its relation to micro-organisms by Raab (1900, 1903), who, obtaining discordant results in attempting to determine the minimal fatal dose of acridine for paramœcia, was finally led to discover that acridine was lethal only in light. Since then much work has been done on this subject by Tappeiner and others (Tappeiner, 1907, 1909), who showed that this property is possessed by many substances, most of which, although by no means all, are fluorescent.

Concentrations of dye and organism, which, in the dark, have no perceptible influence, at once become lethal when exposed to visible light. The addition to the organism of dye solution which has previously been exposed is without influence. Likewise, unexposed dye is without effect when added to previously exposed organisms. In order to obtain photodynamic sensitization simultaneous exposure of organism in presence of sensitizer is essential. Generally speaking the sensitizers are specific. Perhaps a direct contact of dye with organismal protoplasm is necessary and a selective adsorption is a basis of the specificity.

Most observers agree that the light emanated by the fluorescent sensitizers is of itself inactive, and that there is no evidence that the sensitizers undergo any permanent change when exposed to light. Clark states that the presence of oxygen is necessary for most of the effects produced by visible light, and that the only light effective is light of the wave-length corresponding to the absorption band of the sensitizer.

Still more significant is a statement made by Noach (quoted by Ellis and Wells, 1925) with regard to the chemical action of light on cells. He believes

(1) that this action takes place through the agency of substances which transfer light energy into chemical energy; (2) that there are two groups of such catalysts, fluorescent organic compounds and salts of certain heavy metals; (3) both serve as carriers of oxygen; and (4) from experiments on chromogens and many plants, that the fluorescent organic compound acts by formation of peroxide.

A consideration of these facts in association with evidence already obtained regarding the nature of the action of ultra-violet rays on micro-organisms leads the writer to suggest that photodynamic sensitizers themselves may act through the agency of hydrogen peroxide or some organic peroxide production, and that the typical sensitizer acts (1) by selective adsorption on to the organism concerned, and (2) by peroxide formation when visible light falls upon the organism-sensitizer combination.

I wish to express my indebtedness to Dr. Cooper and Staff of the Light Department of the Leeds General Infirmary, who, without restriction, gave me full use of their valuable apparatus, often, I am sure, at considerable inconvenience to themselves.

My thanks are also due to Dr. B. S. Platt for his assistance in the course of this investigation.

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BILIRUBINÆMIA IN MALIGNANT TERTIAN MALARIA AND BLACKWATER FEVER.

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THE application to malaria of Van den Bergh's test for bilirubin in the circulating plasma with a view to obtaining information on the intravascular phenomena of that disease was obviously indicated. In malaria, whatever be the variety of parasite, there exists at the moment of sporulation a destruction of the infected corpuscles. This destruction results in a degree of hæmoglobinæmia, which must in turn produce an increase of bilirubin. That this is likely is evidenced by the icterus, which is so common an accompaniment of the disease. It is, however, necessary to determine whether that icterus is produced solely as a result of excessive blood-cell destruction, or whether it is partly due to impaired hepatic function, since we have evidence in the frequent enlargement of the liver in the disease that it is affected by the pathological processes of the disease. It is necessary, in other words, to determine whether the bilirubinæmia which exists is due to bilirubin of the immature type, produced by the reticulo-endothelial system in excess of the "handling" capacities of the hepatic cells, or whether it is due to bilirubin of the mature variety which has been reabsorbed back into the blood as a result of an obstructive lesion somewhere in the biliary system distal to the actual hepatic cell.

Gordon Thomson (1924), in the report of his researches into the ætiology of blackwater fever in this territory, came to the conclusion that the disease was due to repeated attacks of malaria in which *P. falciparum* was the infecting agent. It seemed pertinent, therefore, to study the degree and nature of bilirubinæmia in the two diseases in any endeavour to prove the one to be a sequel or complication of the other. In addition it was desired to obtain information on the site of hæmolysis in blackwater fever, so that Barratt and Yorke's (1909) claim that hæmolysis took place in the blood-stream could be compared with that of Plehn (1920), who considers that it is extravascular and renal in origin. If the Van den Bergh reaction gave indirect positive results in the disease it could be logically concluded that hæmolysis was intravascular.

That a positive Van den Bergh reaction could be anticipated in blackwater fever was inferred from the fact that jaundice is a well-marked and definite accompaniment of the disease. It was, however, difficult to foretell whether a direct or indirect positive result would be obtained, since doubt exists as to the site of the hæmolysis, and because of the fact that post-mortem examination

of cases dying from the disease reveals the fact that the gall-bladder is distended with granular inspissated bile. Thus the possibility of an obstructive type of jaundice had to be kept in mind.

With these objects in view, the investigation included an examination of bilirubinæmia in normal, malarial and blackwater cases. In the pathological series the patients, in the majority of cases, were inmates of Salisbury Hospital, although specimens of blood from blackwater fever cases were also obtained from several cases in outlying districts.

There is no necessity to comment on the results obtained in normal cases, which were included to ensure that the reagents and technique could be depended upon to give normal results in such cases. The degree of bilirubinæmia found was invariably within normal limits.

The malarial patients were all suffering from *Plasmodium falciparum* infections.

The technique employed in the investigation was based on the recommendations of McNee and Keefer (1925). The tests were performed on oxylated plasma whenever possible. The qualitative test employed was that of Lepehne (1920–21). In the quantitative estimation the method of Thannhauser and Andersen (1921) was followed. Whenever possible at least 2 c.c. of plasma were used, in order that an adequate amount of fluid should be available for the colorometric estimation, which was made with a Klett biocolorimeter.

The colour standard first employed was an ethereal solution of iron rhodonate, but this was later replaced by an aqueous solution of anhydrous cobaltous sulphate, which was found to give more satisfactory results.

While no difficulty was experienced in the reading of the results in malarial infections, the same cannot be said of blackwater fever cases. It was sometimes exceedingly difficult to read the results of both the qualitative and quantitative tests. McNee and Keefer point out that hæmolysis in serum interferes with the colour reaction, and there can be no doubt that the difficulty found in the present instance was directly dependent upon the degree of hæmoglobinæmia. The addition of the reagents employed in the test results in the formation of acid hæmatin, and this is soluble in the alcoholic solution which contains the azo-bilirubin in the quantitative test. In cases where the degree of hæmoglobinæmia was high it was impossible to estimate the number of units of bilirubin present, although by comparison with a control test in which sulphanilic acid was added alone to the plasma, it was possible to state that the test was strongly positive. In cases in which the plasma contained only small amounts of hæmoglobin, the colours of the test and standard fluids could not be exactly matched. As will be seen from the results, however, the degree of bilirubinæmia in blackwater fever is such that the error introduced in this way can be looked upon as being of minor importance. This, taken in conjunction with the fact that the sponsors of the Van den Bergh reaction have never claimed that the test is quantitatively accurate, but prefer to look upon it as an estimation which merely gives comparable expression to the degree of bilirubinæmia present in any given case to the physiological normal, can be held as justification for regarding the results as substantially correct.

In addition to the Van den Bergh reaction, the qualitative test introduced by Fouchet was also performed on the plasma of all cases. Here again the presence of blood-pigment in the plasma interfered with colour development. This test, while not as valuable as the Van den Bergh, can be accepted as a delicate confirmatory test of the presence of bilirubinæmia.

The testing of the plasma was done as soon as possible after the withdrawal of the blood. This was necessary because it was found that allowing plasma to stand overnight interfered with the rate and degree of colour development. In the case of those specimens sent in from remote districts, it was, of course, impossible to avoid delay, and the number of units recorded in these specimens is probably lower than would have been the case had these specimens been examined in the ordinary way.

BILIRUBINÆMIA IN MALIGNANT TERTIAN MALARIA.

Table I gives the results obtained in the examination of 30 cases of *Plasmodium falciparum* infection in Europeans. The examinations were carried out as soon as the diagnosis had been made by microscopical examination of blood-smears.

It will be seen that 29 out of the 30 cases showed a degree of bilirubinæmia above normal limits. The one exception was a very early case of malaria, mild clinically and with only a few parasites in the blood.

As regards the degree of bilirubinæmia, 4 cases showed less than 1 unit, 12 between 1 and 2 units, 5 between 2 and 3 units, 3 between 3 and 4 units, 5 between 4 and 5 units, and 1 above 7 units. This last case, however, was ultimately shown to be suffering from schistosomiasis in addition to malaria. There was hepatic enlargement, and the occurrence of a direct positive result indicated that an obstructive lesion was present.

In cases where hepatogenous and hæmatogenous jaundice exist at the same time it is difficult to state how great a part is played by either in the final picture. This case, then, is not strictly comparable to the remainder of the series. The degree of bilirubinæmia in the Rhodesian cases tends to be higher than in the cases described by Kingsbury, who made a comprehensive study of the subject (1926), but differences in technique and the chemicals used may easily account for this. The table undoubtedly confirms Kingsbury's finding that high bilirubin values are obtained in malignant tertian malaria.

As regards the effect of anti-malarial treatment on the values obtained, Kingsbury has pointed out that the immediate effect of oral quinine in malignant tertian fever is to cause an increase in the degree of bilirubinæmia, and that this rise may be observed for about 24 hours. The ultimate effect, however, is to cause a decrease in the amount.

Considerable difficulty attended the investigation of this problem in the present instance. The patients were not under the sole control of the observer, and, moreover, in the majority of cases had been taking quinine on their own initiative before admission to hospital.

In Cases 1 to 5, however, previous quinine treatment can be definitely excluded. These were all first attacks of malaria in adults recently arrived in the colony.

TABLE I.

Case No.	Disease.	Fouchet.	Van den Bergh. Direct.	Van den Bergh. Indirect.	Anti-malarial treatment.
1	M.T. malaria	0	0	0·25	*Nil*
2	,,	0	0	0·6	,,
3	,,	0	0	0·75	,,
4	,,	(+)	0	0·9	,,
5	,,	(+)	0	1·0	,,
6	,,	(+)	0	1·0	Q
7	,,	(+)	0	1·1	*Nil*
8	,,	(+)	0	1·1	Q
9	,,	(+)	0	1·2	Q
10	,,	(+)	0	1·2	Q
11	,,	+	0	1·4	Q
12	,,	+	0	1·4	Q
13	,,	+	0	1·5	Q
14	,,	+	0	1·5	*Nil*
15	,,	+	0	1·6	Q
16	,,	+	0	1·8	Q
17	,,	+	0	2	Q
18	,,	+	0	2·2	*Nil*
19	,,	+	0	2·5	Q
20	,,	+	0	2·8	*Nil*
21	,,	+	0	2·8	Q
22	,,	+	0	3·2	P
23	,,	+	0	3·2	Q
24	,,	+	0	3·5	P
25	,,	+	0	4	*Nil*
26	,,	+	0	4	Q
27	,,	+	0	4·6	P
28	,,	+	0	4·7	P
29	,,	+	0	4·8	Q
30	Schistosomiasis	+	+	7·2	Q

The following symbols are used

0 = no colour development. + + = marked colour development.
(+) = faint colour development. Q = quinine treatment.
+ = distinct colour development. P = plasmochin treatment.

Cases 7, 14 and 18 were patients who had previously suffered from blackwater fever, and, in all, quinine had played an important part in the genesis of the disease. All had suffered from many previous attacks of malaria. Case 7 had been twice in hospital previously with malaria. On the first occasion the administration of 10 gr. of quinine hydrochloride was followed by hæmoglobinuria a few hours later. On the second occasion an attempt was made to control the malarial infection by novarsenobenzol and sodium methylarsenate. For 7 days malarial parasites were demonstrable, and on the eighth day 10 gr. of quinine hydrochloride were given at 9 a.m. At 10 a.m.

the patient developed hæmoglobinuria. In the present instance no quinine treatment was given in these cases.

In Cases 20 and 25 quinine was withheld because of the patients' idiosyncrasy to the drug.

In Cases 1 to 5 the degree of bilirubinæmia was low; the blackwater susceptibles showed figures varying from 1·1 to 2·2, while the units present in the patients of the last group were 2·8 and 4 respectively. As regards the degree of infection in these cases, in the last two heavy infection of the blood was present, but in the remainder the infection was slight or moderate.

One cannot assume from these figures that patients susceptible to blackwater fever and suffering from malignant tertian malaria show an increased bilirubinæmia, compared with non-susceptibles, in the absence of quinine treatment.

In those patients of the series who had received quinine treatment the degree of bilirubinæmia tended on the whole to be greater. As the examination was invariably done within 24 hours of the commencement of adequate treatment, this result in the light of Kingsbury's work was to be expected.

That the ultimate result of quinine treatment was to restore the degree of bilirubinæmia to normal limits was proved by the examination of several cases.

The examinations were done at intervals, and the following table shows the results obtained:

TABLE II.—*Units of Bilirubin Present in M.T. Infections Treated with Quinine.*

		Days.						
		1.	2.	3.	4.	5.	6.	7.
1.	0·75	1·0	0·6	0·4	..	0·4	..	..
2.	0·9	1·3	1·0	..	..	..	0·4	..
3.	1·2	2·0	1·0	..	0·3	..	..	0·3
4.	2·3	2·7	1·2	..	0·6	..	..	..
5.	3	4·2	2·5	..	..	0·25	..	..

There is, therefore, justification for the belief that one of the results of quinine administration in malaria is the production of an intravascular hæmolysis. It is reasonable to assume that it is the infected corpuscles which are destroyed, since, provided the dosage is sufficient to control the malarial infection, this hæmolysis does not recur. It is particularly important to remember this hæmolytic effect in view of the part that quinine sometimes plays in the ætiology of blackwater fever.

Cases 22, 24, 27 and 28 had been undergoing treatment with plasmochin for 24 hours before the examination was made. They had received 0·1 gm. of the drug, and in all cases parasites were still present in the peripheral blood. The degree of bilirubinæmia was fairly high, but comparison has yet to be made of the relative effects of plasmochin and quinine on the production of bilirubinæmia.

The observations in this section may be summed up by saying that, in

malignant tertian malaria, evidence of intravascular hæmolysis has been demonstrable by the considerable degree of bilirubinæmia which accompanies the disease. Further, the greater degree present after quinine treatment indicates that one of the immediate effects of quinine is to increase hæmolysis, although the effect of continued treatment is ultimately to reduce the bilirubinæmia to normal limits.

Since this improvement is synchronous with the abatement of the clinical symptoms of the disease and the disappearance of parasites from the peripheral blood, it can be reasonably assumed that the initial hæmolysis after quinine is due to lysis of infected corpuscles, and that the ultimate reduction of the bilirubinæmia indicates a failure of the parasite to infect further cells.

The Fouchet test, as can be seen, gives little information beyond demonstrating an excess of bilirubin, and is not so delicate as the Van den Bergh reaction.

BILIRUBINÆMIA IN BLACKWATER FEVER.

The essential feature of blackwater fever is the excretion of hæmoglobin by the kidney, but the clinical symptoms which accompany this phenomenon and the clinical course of the disease may vary to a considerable extent. In any study of the disease it is necessary to classify the cases according to the prominent symptoms, which may be determined by the severity of the hæmolytic process, by the ability of the kidney to dispose of the products of that process, or by a combination of both.

In Southern Rhodesia the majority of cases, after a period of hæmoglobinuria, which may last from one to three days, during which no symptoms of suppression are evident, make an uninterrupted recovery. The fatal cases generally fall into one or two categories. The first type, in which erythrocyte destruction, as shown by blood-count, is tremendous, collapse and die in about 48 hours. No indications of suppression accompany this type, and, indeed, polyuria may be present. In the second type suppression is the cardinal symptom. After the passage of one or two specimens of urine containing hæmoglobin, the patient develops more or less complete anuria, which may last for as long as ten days and which generally results in death.

It was important to determine to what extent bilirubinæmia was influenced by the clinical type developing, and therefore the cases in this section have been classified according to the clinical course of the disease. The classification is as follows:

Type 1: Cases with malignant tertian malaria in whom extremely mild hæmoglobinuria developed.

Type 2: Cases with definite hæmoglobinuria of moderate severity; no suppression; recovery.

Type 3: Cases with severe hæmoglobinuria; no suppression; death in 24–48 hours.

Type 4: Cases with severe hæmoglobinuria; almost immediate suppression; anuria lasting 7–10 days; death.

Type 5: Cases with long-continued hæmoglobinuria lasting up to a week; no suppression; recovery.

The results of the *Fouchet* and *Van den Bergh* tests on these five types

of cases are given in Table III. Unless where stated the examination was performed within 24 hours of the commencement of the attack.

TABLE III.

Type.	Case No.	M.T. parasites at time of examination.	Fouchet.	Van den Bergh.		Time of examination.	Recovery or death.
				Direct.	Indirect.		
Group I	1	+	++	0	6	1st day	Recovery
	2	+	++	0	6	,,	,,
	3	+	++	0	6·4	,,	,,
	4	+	++	0	6·8	,,	,,
Group II	5	+	++	0	10·4	,,	,,
	6	—	++	0	8·5*	?	,,
	7	—	++	0	6·8*	?	,,
	8	—	++	0	5·2*	?	,,
	9	+	++	0	16	1st day	,,
	10	—	++	0	8·2	,,	,,
	11	—	++	0	9·1	,,	,,
	12	—	++	0	7·4	,,	,,
	13	—	++	0	11·6	,,	,,
Group III	14	—	++	0	20·8	?	Death
	15	+	++	0	6·5	1st day	,,
	16	—	++	0	13†	,,	,,
Group IV	17	—	++	++	18·4†	3rd day	,,
	18	—	++	++	27	4th day	,,
	19	—	++	++	59	,,	,,
Group V	20	—	++	0	6·2	1st day	Recovery
	21	—	++	0	11·4	,,	,,

* Specimens in which more than 24 hours had elapsed between withdrawal and examination.
† Approximate values. Excess of free hæmoglobin in plasma.

Before discussing these results it would seem necessary to give a brief *résumé* of the most important points in these cases.

The cases in Group I present histories which are worthy of considerable attention.

Case 1 was admitted to hospital with malaria and malignant tertian rings were found in the peripheral blood. He was treated with plasmochin, 0·1 gm. daily for 5 days. Parasites disappeared from the blood on the third day of treatment. Two days after the completion of the plasmochin course, the patient having been afebrile for a few days, he was given 5 gr. of quinine hydrochloride at 6 p.m. During the night the patient complained of backache and headache, and at 4 a.m. the following morning he passed about 350 c.c. of urine containing hæmoglobin. Urine passed at 7 a.m. was free from blood-pigment. The examination of the plasma at 10 a.m. gave the result recorded. During the height of his malarial attack the number of units present was 3·5. No further hæmoglobinuria occurred.

Case 2 was suffering from malignant tertian malaria and was undergoing quinine treatment. He exhibited no symptoms suggestive of blackwater fever, but examination of the plasma on the second day in hospital revealing what was considered a high degree of bilirubinæmia, urine passed during the previous 24 hours was tested for blood-pigment.

The benzidine and guaiacum tests both gave positive results, but not sufficient hæmoglobin was present to produce absorption bands when a Zeiss direct-vision spectroscope was employed. The sediment did not contain erythrocytes or pus-cells. The urine on the following six days was free from hæmoglobin.

Case 3 was a patient who had suffered from hæmoglobinuria on two previous occasions. In the present instance, while suffering from malignant tertian malaria, owing to the fear of blackwater supervening he was admitted to hospital. Examination of the plasma on the day of admission having revealed 6·4 units of bilirubin to be present, the urine was collected and tested in 24-hour specimens. Urine passed on the first day in hospital was positive for blood by the benzidene and guaiacum tests, but no absorption bands were noted. The sediment was negative for erythrocytes. No blood-pigment was found in specimens passed during the seven following days.

Case 4 was similar. He was admitted as a case of blackwater fever, one specimen of urine passed several hours before admission, it was stated, having contained hæmoglobin. Malignant tertian parasites were found in the peripheral blood, and urine passed during the first day in hospital was dark, but to naked-eye inspection could not be considered more than "smoky." Blood-pigment was proved to be present by the benzidene and guaiacum tests. Spectroscopical examination was again negative, and the sediment showed no erythrocytes or pus-cells. Blood-pigment was present in the following day's urine, but subsequently the patient remained free until discharge.

In these four cases, with the exception of the first, in which definite hæmoglobinuria was evident, the urine contained such small amounts of hæmoglobin that the probability is that in ordinary circumstances the specimens would have been regarded as merely dark febrile urines. Blacklock (1923) believes that "short of blackwater fever which represents a gross hæmoglobinuria, there must be many degrees of hæmolysis, hæmoglobinæmia and slight hæmoglobinuria produced by the same agencies as produce blackwater," and that "hæmoglobinuria occurs frequently in the tropics without being observed." The histories of the above four cases would seem to prove the truth of Blacklock's contention.

In Group 2, Cases 5 to 13 call for no special comment. All were typical cases of the disease, exhibiting the usual symptomatology, progressing to a favourable termination. Two cases showed malignant tertian infection when examined.

In Group 3 the main feature of the cases was the rapidity with which death ensued. In all there was no indication of suppression, and in fact Cases 15 and 16 had polyuria.

These cases are regarded as cases in which the blood destruction itself, producing a condition of intense anæmia, was responsible for the fatal issue.

Case 16 may be given as typical of the group. The patient gave a history of many attacks of malaria in the previous three months. Blackwater fever developed suddenly at 4 p.m. one afternoon. It was accompanied by a rigor and backache. Jaundice was not particularly marked. He was admitted to hospital the following morning, and at 11 a.m. was obviously much worse. Hiccough and vomiting were distressing symptoms, jaundice was intense, and the patient was semi-comatose. The examination was made at this time. The condition rapidly became worse, and the patient died at 6 p.m. that evening. During the period in hospital 700 c.c. of very dark urine were passed, and at post-mortem the bladder contained about 500 c.c. more. The blood-count on admission was 1,260,000 erythrocytes per c.c. The plasma contained both methæmoglobin and oxyhæmoglobin, which rendered the reading of the Fouchet and Van den Bergh tests exceedingly difficult. The figure given in the table can only be regarded as an approximate value.

In Group 4 the factor responsible for death was suppression. Case 17 was admitted to hospital two hours after passing urine containing hæmoglobin. During the first 24 hours about 250 c.c. of very black urine were passed, but thereafter suppression was almost complete, only about 20–30 c.c. being passed daily. The urine passed on the last three days was clear but went solid on boiling, and was much more like a serous exudate than anything else. Jaundice was intense after the first 12 hours for a period of 5 days but gradually lessened, and at the time of death, after 10 days' illness, it was absent.

Cases 18 and 19 are essentially similar in their symptomatology. At the commencement

of the attack both passed a large amount of black urine in the first 12 hours. Thereafter there was more or less complete anuria until death, which in Case 18 occurred on the 8th day, and in Case 19 on the 11th day. These cases, both in remote country districts, were seen on the 4th day; jaundice was intense—in fact the writer has never observed it of greater degree, and it continued so until death. Case 18, just prior to death, passed about 150 c.c. of dark green urine, which was found to contain no hæmoglobin, but bile in enormous amounts.

Case 17, while similar as regards anuria to Cases 15 and 16, differed in that the initial jaundice disappeared. This will be further commented on when the results of the tests for bilirubinæmia are discussed.

Cases in Group 5 were chiefly characterized by the fact that hæmoglobinuria persisted for a considerable period. In Case 20 the urine contained hæmoglobin in considerable quantities for seven days. Jaundice was of moderate degree throughout, and gradually disappeared when the urine became clear. The quantity of urine passed daily was normal. Anæmia was found at the end of the period, but the patient gradually recovered.

Case 21 was similar, hæmoglobinuria persisting for 9 days. The daily amount excreted gradually became less. Anæmia was again an important feature of the case (1,910,000 erythrocytes when hæmoglobinuria ceased), but recovery was uneventful.

While, however, it has been thought necessary to classify the cases examined in this manner, the results of the Van den Bergh and Fouchet tests show that there are certain features which are common to all. Thus all show a degree of bilirubinæmia which is much higher than that which exists in cases of malignant tertian malaria. Moreover in all but two of the cases an indirect positive result was obtained. This indicates that the bilirubinæmia, initially at least, is due to the abnormal hæmolysis which occurs in the disease, and represents an increased effort on the part of the normal mechanism of disposal to get rid of the excess of circulating hæmoglobin. The fact that bilirubinæmia of this type exists can further be said to prove the truth of Barratt and Yorke's theory that hæmolysis is intravascular, for no such phenomenon would accompany an extravascular hæmolysis in the renal tubules.

The degree of bilirubinæmia found varied from 5·2 to 57 units. The smaller values were mostly obtained in cases in which the degree of hæmoglobinuria was exceedingly slight, while the higher values were present in cases in which suppression of urine was the outstanding feature of the disease. It is noteworthy that values obtained in the four cases in Group I agreed to a remarkable degree, all being between 6 and 7 units. It would seem that if the hæmoglobinæmia is such that it can be indirectly represented by a bilirubinæmia of less than 6, the renal mechanism of disposal is not called into play. At or about this value, the hæmoglobin begins to appear in the urine. It is perhaps unwise to lay down such a numerical limit, since the figure may vary with the chemicals and technique employed in the test, but it can be fairly assumed from the results that a definite limit does exist.

If these cases alone were considered, it might be thought that hæmoglobinuria occurred only when the reticulo-endothelial system could not dispose of an excess of hæmoglobin above an amount which, converted into bilirubin, represented a definite and constant value. In other words, renal excretion of hæmoglobin might be regarded as being secondary to excretion by the normal route.

That this is not so is evident when the other cases are considered. Excluding results on plasma in which examination was delayed for more than

twenty-four hours, several cases, in which the degree of hæmoglobinuria was considerable, gave bilirubin values which approximate closely to the values obtained in these mild cases, but the vast majority show figures much in advance. Thus the limit of capability of the reticulo-endothelial system cannot be put down at as low a figure as at first might be suggested by the results in the milder cases, and, if it is capable of converting hæmoglobin into bilirubin to such an extent as was found in the severer cases, there must be some factor independent of the reticulo-endothelial system which determines the appearance of hæmoglobinuria.

The fact that high indirect values have been found in other diseases in which hæmolysis occurs without hæmoglobinuria must emphasize this fact. The reticulo-endothelial system must therefore be regarded as an important factor in the disposal of the free hæmoglobin, but must be divorced from any connection with the appearance of hæmoglobin in the urine.

We are therefore led to the conclusion that hæmoglobinuria depends upon the rate and extent of the hæmolytic process, and that once a certain renal threshold value for hæmoglobin has been reached, excretion of hæmoglobin by the kidney begins. Manson-Bahr and Sayers (1927) consider that the appearance of hæmoglobinuria depends more upon the rapidity of the hæmolysis than the degree. They point out that the erythrocyte count may fall from five million to one million without hæmoglobinuria, provided the rate of fall is such that the reticulo-endothelial system can deal with it. The sudden hæmolysis which occurs in malaria, however, is unaccompanied by hæmoglobinuria, and obviously both the rate and extent of hæmolysis must enter equally into the question. Once sudden intravascular hæmolysis, of sufficient extent to be above the renal threshold for hæmoglobin, has taken place, then the kidney and the reticulo-endothelial system act simultaneously in endeavouring to rid the plasma of hæmoglobin. That the renal route of excretion comes into play at once in these cases is shown by certain experiments which were conducted on rabbits. When an aqueous solution of hæmoglobin was introduced intravenously, three of the experimental animals passed urine containing hæmoglobin in less than five minutes. That the reticulo-endothelial system begins to function at the same time can be assumed from the fact that, if cases are seen immediately after the appearance of hæmoglobinuria, jaundice may not be evident, but on the following day is generally a prominent symptom.

The extent and duration of the bilirubinæmia must, however, depend upon the rapidity and extent of the excretion of hæmoglobin by the renal route, and also upon the capability of the body to dispose of the additional bile formed. That this is so is illustrated by the cases in which suppression existed. In two of these cases a direct positive Van den Bergh reaction was obtained, which suggests that an obstructive type of jaundice had been superimposed upon the hæmolytic type originally present. That this may occur is highly probable when the post-mortem condition of the gall-bladder and liver in cases dying from the disease is recalled. There is invariably marked distension of the gall-bladder and of the bile-ducts with thick tarry inspissated bile, and in some cases the bile is almost solid. In these circumstances the probability of an obstruction to the flow of further bile can readily be imagined. This must be

more likely to happen when, owing to the blocking of the renal tubules and the consequent inability of the body to dispose of the hæmoglobin by the renal route, the whole burden of excretion is thrown on to the reticulo-endothelial system. In the two cases in which positive direct Van den Bergh results were obtained, the patients were undoubtedly suffering from both renal and biliary obstruction. The remaining case in this group indicates, however, that this contingency need not necessarily occur, and suggests the possibility that, provided biliary obstruction does not occur, the free hæmoglobin may be got rid of entirely by the reticulo-endothelial system. In this case on the first day it was impossible to estimate the number of units of bilirubin present owing to the high degree of hæmoglobinæmia, which, estimated by the Newcomer method, amounted to ·8 gm. Hb. per 100 c.c. plasma. Two days later the amount had fallen to 0·69 gm. per 100 c.c., and an approximate estimation of 18·4 units of bilirubin was made. Further examination was unfortunately impossible, but the decline in the percentage of hæmoglobin free in the plasma during these two days, the fact that no hæmoglobin was passed in the urine, and that the jaundice eventually disappeared, conveys the impression that an unobstructed biliary system was able to remove the excess of hæmoglobin present.

THE MECHANISM OF EXCRETION OF FREE HÆMOGLOBIN.

From a consideration of the results obtained in the investigation of malarial and blackwater cases, it would appear that the appearance of hæmoglobin in the urine depends entirely upon the concentration of hæmoglobin in the plasma. In the malaria cases, hæmolysis, though sudden, is relatively slight, the threshold value for hæmoglobin is not reached, and the reticulo-endothelial system is able to dispose of the excess present. When, however, the concentration of hæmoglobin exceeds the threshold value immediate excretion by the kidneys occurs, while the reticulo-endothelial system at the same time becomes active in an independent effort to remove the hæmoglobin. The extent to which it is called upon depends upon the rate and degree of renal excretion, and, in certain cases, where anuria occurs it may have to undertake the whole duty. In these cases there is the possibility that obstruction of the biliary system may supervene, and that a complete cessation of the whole excretory mechanism may occur. When hæmolysis is such that the renal threshold value is not reached, the activity of the reticulo-endothelial system, as evidenced by the production of bilirubin, is probably in direct proportion to the extent of the hæmolysis. The cases in the present series, which have been regarded as threshold cases, suggest that this is so, for with a degree of hæmoglobinæmia, as reflected by the degree of hæmoglobinuria, practically the same in all, the degree of bilirubinæmia was likewise similar. What probably happens in such cases is that the small excess over the threshold value is excreted without difficulty by the renal route, while the remaining portion is then converted into bilirubin, and the fact that the degree of bilirubinæmia was so uniform in these cases points to a quantitative relationship between it and the amount of hæmoglobin to be disposed of.

We can thus assume further that the renal threshold value for hæmoglobin

does not vary with the individual, but can be represented by a uniform constant value.

The degree of bilirubinæmia seen in these cases cannot be regarded as representing the limit of output of the reticulo-endothelial system above which hæmoglobinuria occurs, for the capacity of the system is much in excess of this, and, in the presence of plasma hæmoglobin above the renal threshold value, both mechanisms of disposal act simultaneously. The determining factor in hæmoglobinuria is the rapidity and extent of hæmolysis.

THE BEARING OF THE RESULTS ON THE RELATIONSHIP OF MALIGNANT TERTIAN MALARIA TO BLACKWATER.

The results do not justify more than a guarded statement on the vexed question of the relationship between these diseases. The information given by Fouchet and the Van den Bergh reactions is limited. They merely tell us that an excess of bilirubin is present in both diseases, and that it is due to excessive hæmolysis. No information can be expected on the nature of the hæmolytic process. The cases in Group A are perhaps the most suggestive as regards the ætiology of blackwater fever, in that they show that undoubted malignant tertian malaria may be accompanied by a hæmoglobinuria so slight as to escape notice. The question at once arises as to whether that hæmoglobinuria can be regarded as blackwater fever. Castellani and Chalmers (1919) would, of course, regard these cases as merely symptomatic hæmoglobinurias, but there is just as much justification for regarding them as mild blackwater fever. It is doubtful whether it is reasonable to elevate the major degrees of any pathological process into separate clinical entities. The manifestations of any disease must range from the minor to the major, and, in a survey of the pathology, it is undoubtedly better to adopt the broader point of view. Adopting this attitude, it can be said that the hypothesis that malignant tertian malaria is an ætiological factor in blackwater fever has certainly not been weakened by the demonstration that mild hæmoglobinuria can occur during the former disease.

SUMMARY.

(1) Examination of 30 cases of malignant tertian malaria and 21 cases of blackwater fever by the Fouchet and Van den Bergh reactions show that an increased bilirubinæmia occurs in both diseases.

(2) Bilirubinæmia in malignant tertian malaria and initially in blackwater fever is due to intravascular hæmolysis, as evidenced by the occurrence of an indirect Van den Bergh reaction.

(3) In blackwater fever an obstructive jaundice may be superadded to the initial hæmolytic jaundice. This has been observed in cases suffering from suppression of urine.

(4) Cases of mild hæmoglobinuria in the course of malignant tertian malaria are reported. Attention was directed to one of these cases by the degree of bilirubinæmia found. The degree of bilirubinæmia was remarkably uniform in these cases.

(5) It is considered that the results justify the assumption that hæmoglobinuria is dependent upon the rapidity and degree of hæmolysis. Below a

renal threshold value for hæmoglobin, the reticulo-endothelial system only is concerned in the excretion of the excess. Above this value both the kidney and reticulo-endothelial system come into play simultaneously. Renal excretion of hæmoglobin is, therefore, independent of the activity of the reticulo-endothelial system.

(6) The bearing of these results on the relationship of malignant tertian malaria and blackwater fever is discussed.

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AN INVESTIGATION OF THE ANTIGENIC PROPERTIES OF FOUR SPECIMENS OF BENCE-JONES PROTEIN OBTAINED FROM CASES OF MYELOMATOSIS.

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SINCE Bence-Jones protein was first described, it has been made the subject of a number of investigations and found to possess certain properties which distinguish it from other proteins. The study of the antigenic properties of the protein has produced information of considerable interest. The earlier work in this field was rendered of little value because the preparations investigated were impure, but it has now been definitely established that the Bence-Jones protein is antigenically different from the proteins of normal human serum (Massini, 1911; Hektoen, 1921; Bayne-Jones and

Wilson, 1922; Hektoen and Welker, 1924). The fact that the various specimens of Bence-Jones proteins have been found to possess very similar chemical and physical properties has led to the assumption that they are identical in structure and composition. Bayne-Jones and Wilson have shown that a consideration of certain aspects of Bence-Jones proteinuria, more particularly its association with different pathological states, suggests that Bence-Jones proteins may not be similar in structure, and they have put forward strong evidence in support of this view. They studied the antigenic properties of twelve preparations of Bence-Jones proteins isolated from five patients suffering from various diseases by means of the precipitin, complement-fixation and anaphylactic (Schultz-Dale) reactions. The results obtained by them led to the conclusion that a number of proteins had been grouped under the term "Bence-Jones proteins," which, though similar, were not identical. By their immunological tests they were able to recognize two, possibly three, groups of Bence-Jones protein. Confirmation of these interesting observations has not yet been forthcoming, and since four preparations of Bence-Jones protein obtained from four cases of myelomatosis were very kindly placed at my disposal by my colleague, Dr. L. F. Hewitt, it was decided to investigate their antigenic properties.

EXPERIMENTAL DATA.

Preparation of antisera.—The Bence-Jones proteins investigated were obtained from the urine of four patients suffering from myelomatosis by the addition of ammonium sulphate to 40-60% saturation, the precipitation being repeated several times. Approximately 4% solutions of the proteins in saline were used as antigens for the production of antisera. In the precipitin reactions the antigens used were freed from lipoid by alcohol-ether extraction.

Antisera were prepared for two of the specimens only, the quantity of the other two being insufficient to allow of this being done in every case. Rabbits were given intravenous inoculations of 3·0 c.c. of the protein solution at intervals of four days. Test bleedings were made after the third and fourth injection, and when a sufficiently high titre had been reached—definite precipitation with an antigen dilution of 1 in 10,000—the animal was bled out. In order to get sera of a sufficiently high titre a number of rabbits had to be employed, as they showed considerable individual variation in their power to produce an antibody to the Bence-Jones protein; some failed to react at all.

Precipitin Test.

The precipitating power of the two antisera for the four antigens was determined, the test being carried out by making dilutions of the antigen in saline, and adding a fixed amount (1 in 20) of undiluted antiserum to each tube. The contents of the tubes were thoroughly mixed and placed in the incubator at 37°C. for two hours, when the first reading was made. They were then placed in the ice chest overnight, a final reading being made the next day. Suitable controls were included in the test. The results obtained are tabulated in Table I.

TABLE I.

Antigen.	Antiserum L. Dilutions of antigen. 1/10.	1/100.	1/1000.	1/10,000.	Antiserum W. Dilutions of antigen. 1/10.	1/100.	1/1000.	1/10,000.
L.	±	+	∓	∓	−	−	−	−
W.	±	∓	−	−	±	+	+	∓
D.	+	±	−	−	−	−	−	−
H.	+	±	−	−	−	−	−	−
Controls.	The antigens alone in saline without the addition of serum, and the two sera in saline remained perfectly clear.							

It will be seen that whereas antiserum W reacted only with its own antigen, antiserum L reacted with all four of the Bence-Jones proteins including W, though more markedly with its own antigen L. This result suggested that antigen W was the purer of the two, and that antigen L contained normal serum protein in sufficient amount to excite the production of an antibody, which reacted *in vitro* with the traces of a similar impurity in antigens W, D and H.

Antiserum L was therefore put up against normal human serum, and found to give a positive reaction with an antigen dilution of 1 in 1000. As Hektoen has shown, these antibodies for the normal serum protein impurities can be removed by absorption, the Bence-Jones antibody being left. Antiserum L was mixed with about 1/5 its volume of human serum protein, and allowed to stand at room temperature for six hours, centrifuged, and the supernatant serum pipetted off. This supernatant was now shown to have no precipitating action on human serum protein, but when tested against the four preparations of Bence-Jones protein gave results little different from the unabsorbed serum (Table I). Thus the wider range of action of serum L as compared with that of serum W cannot be explained by the existence of the impurity in the former.

The results show that there are antigenic differences between different specimens of Bence-Jones proteins, and that the antigenic "structure" of some may be more complex than that of others.

These results, therefore, confirm those of Bayne-Jones and Wilson.

CONCLUSION.

An examination of the antigenic properties of four specimens of Bence-Jones protein has shown at least two of them to be different.

My thanks are due to Dr. S. P. Bedson for his constant interest and help in this work, and to Dr. L. F. Hewitt, who kindly placed the four preparations of Bence-Jones protein at my disposal.

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A CONTRIBUTION TO THE THERAPY OF ACUTE INFECTIONS.

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THE experiment to be detailed below produced evidence which indicates that the subcutaneous administration of an extract of brain tissue exerts a beneficial effect on the process of immunization. The results also show that the treatment with brain extract tends to lessen the mortality of animals which have been subjected to serious infections.

The theory which suggested brain extract as a therapeutic agent was based on the view that the spleen might play an important part in the elaboration of immune substances. For this there is, it is true, little direct evidence, but the work of Isaac and Billing, Murata, Von Skramlik and Huenermann, and of Aschoff (1925) as well as of Ledingham (1926) has indicated that the reticulo-endothelial system is probably the source of immune substances, and in that system the spleen played no inconsiderable part.

The normal splenic pulsations, which occur at the rate of one a minute (Roy, 1880–82), may be regarded as one indication of functional activity in that organ. Any acceleration of the rate of these pulsations may be conceived as representing an increased functional activity with a consequent increase in the rate of elaboration of immune substances.

Schafer and Moore (1896) have shown that under conditions of experiment the rate of pulsation of the spleen could be markedly influenced by the administration of certain agents. On the one hand, atropine had a definitely inhibitory effect, while, on the other hand, a watery extract of brain was most active in producing an accelerated rate of pulsation.

That these substances might influence the course of infections has been submitted to a preliminary test in a number of experiments. It is not suggested that the beneficial effects which appear to accrue from the use of brain extract are necessarily explained by those theoretical considerations which have been advanced, but they appear to be sufficiently definite to merit recording, and to stimulate further work on the same lines.

The observations fall into two main groups:

1. On the effect of injections of brain extract and of atropine on the immunization of rabbits, as indicated by the agglutination titre to *B. typhosus*.

2. On the effect of injections of brain extract in diminishing the mortality of mice from inoculations with pneumococcus type I.

PART I.

Twelve adult rabbits were inoculated subcutaneously with a saline emulsion of *B. typhosus*—a 24-hour growth killed by heat and standardized to 3000 million to 1 c.c.; 4 were regarded as controls and received no other injections;

4 others received in addition subcutaneous injections of a solution of atrop ne sulphate, 0·000065 gm. in 1 c.c., while the remaining 4 received injections of an aqueous brain extract.

The aqueous brain extract was prepared by washing the brain of a healthy ox in normal saline. It was then cut into small pieces, rinsed in normal saline, and boiled in about twice its volume of distilled water for an hour and a half. After several filtrations the opalescent filtrate was sterilized by the intermittent method.

One further point of technique requires explanation. The time between injections as shown in Tables I and III varies. Table I represents a repetition of a previous experiment in which injections were given every second or third day, while in the group shown in Table III the usual interval was allowed.

During immunization by the method described above the rate of development of agglutination in the sera of the rabbits was observed, and the results are tabulated in Tables II and IV.

TABLE I.

Animal.	Dose in c.c. and days under treatment.																					
	1.		3.		6.		8.		10.		15.		17.		20.		22.					
R. 35 . .	B.T. 0·1		B.T. 0·2		B.T. 0·3		B.T. 0·4		B.T. 0·5		B.T. 1		B.T. 1·2		B.T. 3							
R. 36 . .	0·1	A. 0·1	0·2	A. 0·2	0·3	A. 0·3	0·4	A. 0·4	0·5	A. 1	1	A. 2	1·2	A. 2	3	A. 3	A. 3					
R. 37 . .	0·1	B. 0·5	0·2	B. 0·5	0·3	B. 1	0·4	B. 1	0·5	B. 1	1	B. 2	1·2	B. 3	3	B. 3	B. 3					

B.T. = Emulsion *B. typhosus*, 3000 million in 1 c.c.
A. = Atropine sulphate, 0·000065 gm. in 1 c.c. normal saline.
B. = Brain extract.

TABLE II.—*Agglutination Titre.*

Animal.	Days under treatment.		
	10.	17.	24.
R. 35	1–100	1–800	1–3200
R. 36	*Nil* in 1–50	1–400	1–800
R. 37	1–800	1–1600	1–256,000

CONTROLS.

One animal (R. 20) received injections of brain extract corresponding to those received by the animals in the main experimental groups. On the first and second days it received 1 c.c. injections, while on the sixth and seventh days the dose on each day was 2 c.c.

TABLE III.

Animal.	Dose in c.c. and days under treatment.											
	1.		2.		6.		7.		11.		12.	
	B.T.		B.T.		B.T.		B.T.		B.T.		B.T.	
R. 38	1		...		2		...		3		...	
R. 39	1		...		2		...		3		...	
R. 40	1		...		2		...		3		...	
		A.		A.		A.		A.		A.		A.
R. 41	1	1	...	1	2	1·5	...	2	3	2	...	3
		A.		A.		A.		A.		A.		A.
R. 42	1	1	...	1	2	1·5	...	2	3	2	...	3
		A.		A.		A.		A.		A.		A.
R. 43	1	1	...	1	2	1·5	...	2	3	2	...	3
		B.		B.		B.		B.		B.		B.
R. 44	1	1	...	1	2	2	...	3	3	3	...	4
		B.		B.		B.		B.		B.		B.
R. 45	1	1	...	1	2	2	...	3	3	3	...	4
		B.		B.		B.		B.		B.		B.
R. 46	1	1	...	1	2	2	...	3	3	3	...	4

TABLE IV.—*Agglutination Titre.*

Animal.	Titre and day under treatment.		
	6.	11.	16.
R. 38	1–200	1–800	1–1600
R. 39	1–400	1–800	1–3200
R. 40	1–400	1–800	1–12,800
R. 41	1–100	1–3200	1–3200
R. 42	1–50	1–400	1–800
R. 43	1–50	1–400	1–800
R. 44	1–1600	1–32,000	1–102,400
R. 45	1–1600	1–16,000	1–204,800
R. 46	1–400	1–25,600	1–102,400

The control experiments were on the following basis:

1. To test a brain extract serum against an emulsion of *B. typhosus* and note if there is any direct effect on the agglutinability of the organisms.

2. A brain extract serum and an anti-typhoid serum of known agglutination titre were mixed in equal parts and their combined agglutination effect on an emulsion of *B. typhosus* was determined.

3. An anti-typhoid serum was tested against brain extract to note if possible any effect brain extract may have on an immunized serum.

Control Results on Sixth Day.

1. Brain extract serum (R. 20) titrated against an emulsion of *B. typhosus* gave no agglutination in 1–20.

2. Brain extract serum (R. 20) and anti-typhoid serum (R. 39, titre 1–3200) titrated against an emulsion of *B. typhosus* gave an agglutination titre of 1–1280.

3. Anti-typhoid serum (R. 39, titre 1–3200) titrated against brain extract gave no precipitation in 1–20.

Control Results on Eleventh Day.

1. Brain extract serum (R. 20) titrated against an emulsion of *B. typhosus* gave no agglutination in 1–20.

2. Brain extract serum (R. 20) and anti-typhoid serum (R. 45, titre 1–16,000) titrated against an emulsion of *B. typhosus* gave an agglutination reaction at 1–8000.

3. Anti-typhoid serum (R. 45, titre 1–16,000) titrated against brain extract gave no precipitation in 1–20.

SUMMARY.

The number of these experiments is not large, but they bear the interpretation that while inoculation of rabbits with *B. typhosus* alone results in a satisfactory production of agglutinins, the immunization process is much accelerated and a much higher titre is reached when brain extract is also injected.

In contrast, atropine sulphate depresses the rate of agglutinin formation.

PART II.

The results recorded from the agglutination experiments showed some relative value. It therefore seemed desirable to have more definite proof that the brain extract created protective properties, and this was attempted by testing whether in the presence of a virulent infection it could prevent or diminish the mortality.

In the preliminary tests, a few rabbits in one group were injected with *Staphylococcus aureus* and those of another group with pneumococcus type I. Those tests gave results which appeared to favour the idea that brain extract had some beneficial effect. However, the number of animals used was too small to render the results sufficient to form a basis worthy of any certain consideration. Further experiments on mice inoculated with pneumococcus type I gave more certain results, as it was possible to work on large numbers.

The experiments on mice were arranged in several series; at first with comparatively small numbers and later with 50 experimental and 50 control animals in each section. 520 mice in all were inoculated, of which 262 were experimentals and 258 controls (Tables V and VI).

In all the following experiments a preliminary test of the virulence of the organism (pneumococcus type I) was made by injecting 0·1 c.c. of a 24-hour serum broth culture in varying dilutions, *i. e.* 1 in 100, 1 in 1000, 1 in 10,000, etc. The dilution, 0·1 c.c. of which killed a mouse in 3 to 4 days, was the standard employed. Only in Experiment 1 was the serum broth culture injected undiluted.

0·1 c.c. were injected subcutaneously, and when animals received two injections at the same time, *e. g.* pneumococcal broth and brain extract, the inoculations were made one on each side.

DISCUSSION.

The experiments detailed in Part I, showing the effect of the subcutaneous administration of an aqueous extract of brain on the development of agglutinins against *B. typhosus* simultaneously injected, are suggestive of an enhanced production of antibodies.

In four animals which received brain extract in addition to an emulsion of *B. typhosus*, the agglutination titre reached a level between 1 in 102,400 and 1 in 256,000, while in the controls, which received only an emulsion of *B. typhosus*, the agglutination titre was between 1 in 1600 and 1 in 12,800. In contrast, those rabbits which were inoculated with atropine, a substance believed to have an action on the spleen opposite to that of brain extract, the agglutination titre was in three out of four cases even lower than that of the controls, and in only one instance was the titre on a level with the controls. On the first reading all were below the control level.

The number of these experiments is too small to bear clear evidence of the beneficial effect of brain extract on immunization, but the results appeared suggestive, and encouraged the development of the same idea in relation to protective action against a dosage with a lethal organism. The experiments on the latter line, which have been carried out mainly on mice, extend over a number large enough to provide significant results. In the various experiments which have been done the number of mice varied to some extent, but in each case the number of protected animals has been compared with an almost equal number of controls. The actual details of administration and dosage also have varied somewhat in the different experiments, but have been the same for protected and control animals in each experiment apart from the administration of brain extract to the former (Table VI). The net result is that of the 520 animals a better rate of survival of the protected animals was obtained to the extent that 133, or 51%, survived after all controls were dead. In the individual experiments, as shown in Table V, there was invariably a significant percentage of the brain extract animals which outlived the controls. Excepting preliminary experiments, the percentage surviving ranged between 26 and 62, while the majority were at about 50.

Generally the experiments were concluded in three weeks, but in several groups the period extended to about eight weeks.

TABLE V.—*Experiments and Number in each Surviving.*

	Mice.				Main experiment.															
	Preliminary.																			
	I.		II.		III.		IV.		V.		VI.		IX.					VII.			VIII.	
	Control.	Experimental.	Control.	Experimental.	Control.	Experimental.	Control.	Experimental.	Control.	Experimental.	Control.	Experimental.	(a) Pneumococci.	(b) Pneumococci and glucose.	(c) Pneumococci and peptone.	(d) Pneumococci and cream.	(e) Pneumococci and brain extract.	Days.	Control.	Experimental.	Control.	Experimental.
Pre-experimental . .	6	6	3	5	12	15	35	48	50	48	46	45	4	4	4	4	4	Pre-experimental.	41	40	50	50
End of 1st day . . .	1	1	3	5	12	15	33	48	46	48	45	45	4	4	4	4	4	1	4	38	26	49
„ 4th „ . . .	0	1	0	4	6	14	23	34	33	46	3	31	2	2	2	2	4	2	0	19	0	31
„ 10th „ . . .	0	0	0	2	2	7	1	22	0	24	0	21	1	2	2	0	4	3	0	2	0	22
„ 21st „ onwards .	0	0	0	0	0	4	0	12	0	19	0	12	0	0	0	0	4	4	0	2	0	12
																		5	0	0	0	12
																		10	0	0	0	8
																		21 onwards	0	0	0	3
Average length of survival in days	1·3	1·6	3·3	10	7	12	6	11·3	5·4	13·4	2·4	10·9	6	11	7	5	22		1	2·5	1·5	5·8
Percentage survival of experimentals after all controls are dead	...	16	...	80	...	26	...	40	..	50	...	57	...	...	...	...	100			48		62

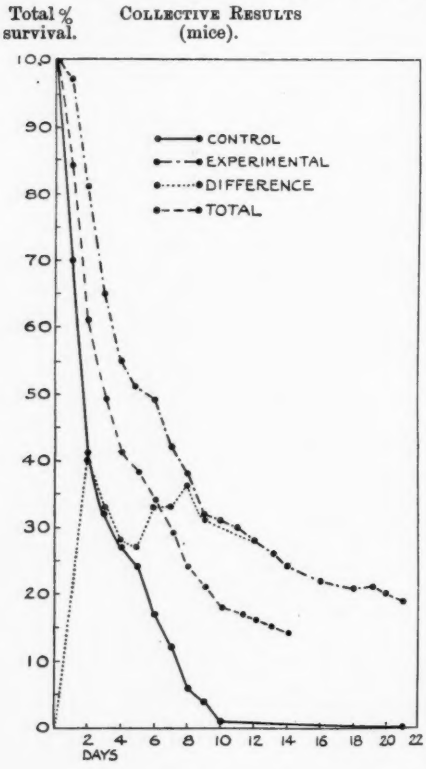
Total number of animals (mice), 520 { 258 controls. 262 experimentals.

TABLE VI.—*Experiments and Amount in c.c. of Aqueous Brain Extract Injected.*

	Mice.									
	I.	II.	III.	IV.	V.	VI.	VII.	VIII.	IX*e*.	
Before pneumococcal injection, 5 days	...	...	...	...	...	...	...	...	...	
,, ,, ,, 4 ,,	0·1	...	...	...	...	...	...	...	...	
,, ,, ,, 3 ,,	0·1	...	...	...	...	...	...	...	...	
,, ,, ,, 2 ,,	0·1	0·2	...	...	...	...	...	...	...	
,, ,, ,, 1 day	0·2	0·4	0·1	0·1	0·1	0·1	0·1	0·1	0·1	IX*b*, *c* and *d* received similar amounts respectively of 10% glucose, peptone, broth or cream at the same intervals.
On day of pneumococcal injection .	0·4	0·4	0·2	0·2	0·2	0·2	0·2	0·2	0·4	
After pneumococcal injection, 1 day	...	0·4	...	...	...	...	...	...	...	
,, ,, ,, 2 days	...	...	0·4	...	0·4	0·4	...	0·4	0·2	
,, ,, ,, 3 ,,	...	...	...	0·4	...	...	...	...	...	
,, ,, ,, 7 ,,	...	...	...	...	0·2	...	...	...	...	
,, ,, ,, 11 ,,	...	...	...	0·2	...	...	...	...	...	

N B.—The brain extract animals of Experiments V and VII were also fed on bread soaked in dilute brain extract. In Experiments VI and VIII a few c.c. of brain extract were added to the water in the drinking bulbs of the brain extract animals.
In Experiments V, VI, VII and VIII the controls each received injections of sterile normal saline similar in amount and on the same day as the experimental animals were inoculated with brain extract.

As the ultimate fate of the surviving protected animals was not pursued indefinitely there is no basis for argument that a complete cure from infection was produced in any case, but it may be noticed that the survival of protected animals after the death of all controls extended in seven sections from two to five weeks; while in the remaining two sections, although the further survival of the brain extract animals can only be measured in days, in comparison with controls the results are suggestive. There therefore appears to be substantial grounds for believing that the injections of brain extract have contributed in some way towards an increased resistance in mice against infection. (See Fig.)



With regard to the way in which the tissue extract produces its beneficial effect, it is hardly possible to make any definite statement at present. One could not be ready to attribute to the brain tissue much therapeutic action, as the tissue had been deprived of most of its solid content by boiling and filtration, and as no analysis has been made of the amount or nature of this solid content of the extract.

The test has been carried out on the basis of the theory which has already been expounded, and the reason for employing this particular substance was that it had been shown under experimental conditions to produce an activating effect on splenic pulsations. This appeared to render the spleen as part of the reticulo-endothelial system accessible to an experimental form of stimulation. I am in no position to say to what extent the original theory accounts for the results obtained, but these results appear to be certainly worthy of recording, and to suggest the desirability of a further exploitation of this form of therapy on experimental lines.

CONCLUSIONS.

1. In a small series of rabbits the subcutaneous injections of a boiled watery extract of brain-tissue have shown an enhanced effect on the process of agglutinin formation against *B. typhosus*.

2. The administration of the same extract to a large series of mice infected with pneumococcus type I has shown a survival of 133 or 51% of a total of 262 after a similar number of control animals were all dead. The controls received only injections of pneumococci. The survival extended over two to three

weeks and in several experiments to from five to eight weeks, when the experiments were concluded.

3. The enhanced effect of brain extract on the process of immunization may be due to the fact established experimentally that the extract of brain stimulates the pulsations and, probably, the functional activity of the spleen.

I am grateful to Prof. Shaw Dunn for his interest, his helpful criticism, and for generously supplementing the available supply of animals; and also to Dr. Twort, Duguid and Downie for their assistance. I am also indebted to the Government Grant Committee of the Royal Society for a grant which in part covered the expense of this investigation.

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ON LOCAL ALTERATIONS OF THE BLOOD-VESSELS OF TAR-PAINTED WHITE MICE.

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THE mode of action of the active principle in the genesis of tar cancer is still quite unknown. Does the tar directly alter the epithelial cell, so as to produce a new type, or is the cancerization a gradual and irreversible adaptation of the cell to a new *milieu*, and the tar cancer a result of the changes of the *milieu* brought about by the tar?

With our present technique very poor information regarding the process of cancerization can be expected from the study of the cytological changes of the epithelial cell. It therefore seems more rational to examine the rather gross alterations of the *milieu* a little closer, and especially the functional and morphological changes of the nervous and vascular tissues.

The *rôle* of the nervous element in the production of tar cancer has been dealt with by Itchikawa, Baum and Uwatoko (1924), Itchikawa and Uwatoko (1924), Itchikawa and Kotzareff (1925), and Cramer (1925), and interesting findings have been reported.

On the other hand, marked local alterations of the blood-vessels and blood-supply have been described by several investigators, such as Dreifuss and Bloch

(1922), Lipschütz (1924), and Itchikawa and Baum (1924[1, 2, 3]). The latter have observed that the tumour formation after tar-painting of the skin of rabbits and mice is always connected with a marked reaction of the vascular system in form of a hyperæmia. In animals that do not respond to the tarring by tumour formation, as rats and guinea-pigs, no such vascular reaction is found. The authors regard the epithelial and vascular responses as parallel phenomena. These findings have been made on ordinary microscopical sections of the tissue.

In this paper I wish to present some observations regarding the local influence of coal-tar on the blood-vessels, obtained with quite another technique. As Rogowicz (1885), Okuneff (1924) and Kusnetzowsky (1925[1, 2]) have shown, a local hyperæmia is easily demonstrable by the use of vital staining dyes. A dilatation of the capillaries is followed by an increased transudation. If the transudate contains a suitable dye, the corresponding tissue will be furnished with greater amounts of the dye than the rest, and accordingly be deeper coloured.

The *technique* employed is as follows: White mice of different sex and varying age were locally epilated with 10% sulphide of sodium. Later they were painted with a potent tar every second day on the epilated area (1·5 × 1·5 cm.), which was sometimes situated in the neck, sometimes in the sacral region, and sometimes in the flank. When the animals were going to be examined, they were injected intraperitoneally with 0·1–0·2 c.c. carmine solution (Kiyono, 1914), and killed 24–48 hours later. Sometimes two or three injections were given with intervals of 24 hours. Animals which died spontaneously were discarded. Some animals were killed by an intravenous injection of china ink. When the animal was dead, autopsy was immediately performed. The skin was removed, spread on a piece of flat cork, and covered with cotton soaked in 10% formalin. After 24 hours' fixation the skin was transferred to 70% alcohol, and the remaining hairs epilated or cut. After passing through the alcohols, some of the skins were transferred to cedar-wood oil *in toto*, and from some the painted area and its surroundings were cut out, and mounted on slides. Numerous ordinary microscopical sections served as controls.

Table I shows a typical record, and the photograph (fig. 1) the result.

TABLE I.—*Mouse, Female, Nine Months Old.*

April 25, 1927	. Both flanks epilated with 10% Na_2S.
„ 26 „	. 0·2 c.c. carmine solution injected intraperitoneally. Right flank painted with tar (" Saxolintjære ").
„ 27 „	. Ditto.
„ 28 „	. Ditto. 8 hours later 2 c.c. china ink injected intravenously. Autopsy.

The photograph clearly shows the dilated blood-vessels, and the deeper colouring of the skin on the right side, corresponding to the painted area.

In this manner the vascular reaction has been followed from the first painting up to the point where warts and papillomas were formed; and it has

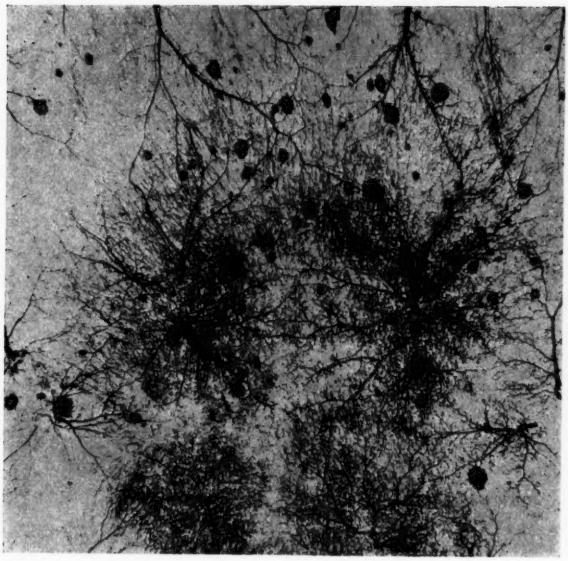

FIG. 2.

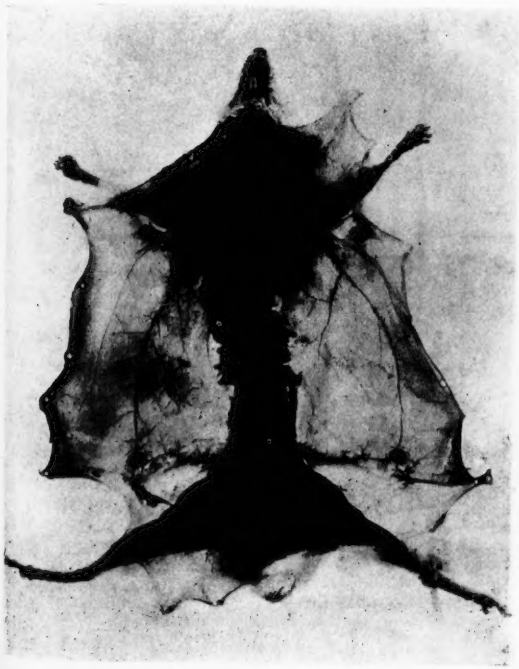

FIG. 1.

FIG. 3.

been shown that the tar produces a dilatation of the blood-vessels, followed by an increased transudation. The hyperæmia lasts as long as the painting is continued, day and night, week after week, and is, as will be seen from fig. 2, strictly local. The picture is a photograph of the skin of the sacral region from a mouse which had been painted 10 times in 22 days and killed by the intravenous injection of 6 c.c. of china ink. With higher magnification (fig. 3) it can be seen that in the painted area practically every capillary is

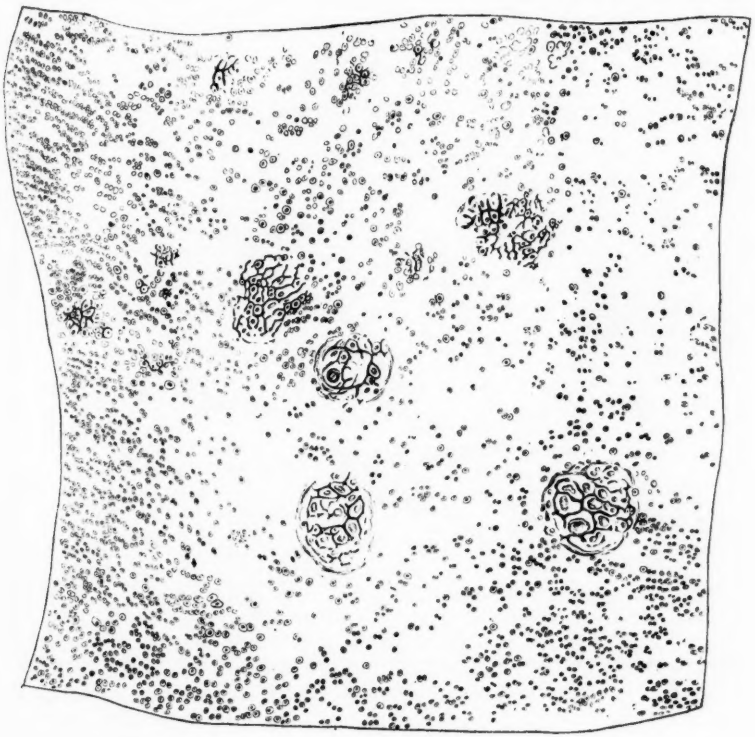

FIG. 4.

filled by the ink, in strong contrast to the surrounding tissue, where only very few capillaries are seen injected. (The dark spots on the pictures indicate ruptured vessels.) When the hyperæmia has been established and the painting continued, we may observe after 12–16 weeks of tarring that on different spots within the treated area one or a few of the capillaries are excessively dilated. The dilatation sometimes reaches a degree that justifies the designation "cavernous vessels." This observation has been recorded by several investigators, as Dreifuss and Bloch (1922), and Lipschütz (1924). But there is one point that, as far as I know, has not been described, and that is

not easily seen except in *in toto* preparations, viz. the close relation which exists between the dilated capillaries and the hypertrophic hair-follicles.

Fig. 4 is a reproduction of a drawing from the skin of a mouse, which had been tarred 55 times in 110 days in the sacral region. The heavy black lines show the dilated capillaries, and the small rings the more or less altered hair-follicles. The three warts were macroscopically visible as tiny spots on the

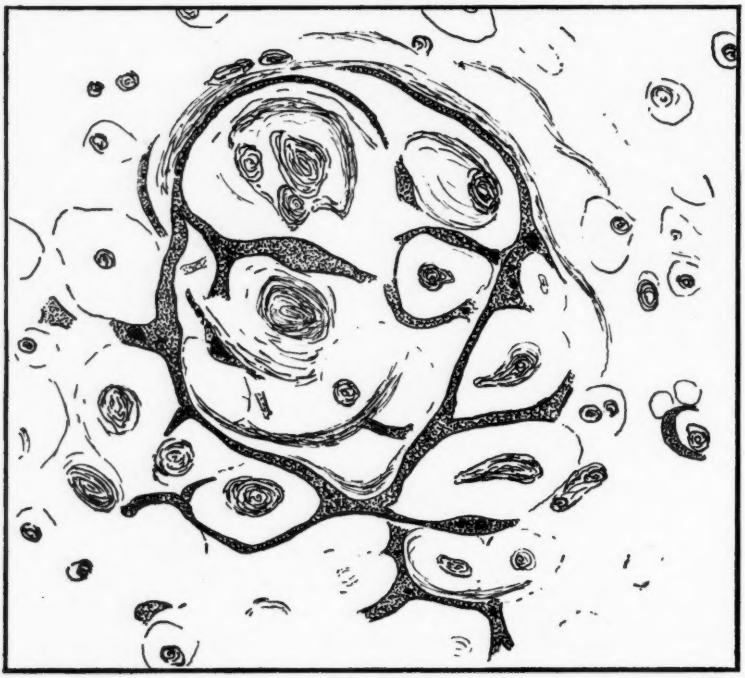

Fig. 5.

skin. But the other groups of hypertrophic follicles, which were not elevated from the surface, could be observed only after the injection.

If we examine the cavernous capillaries more closely in carmine-coloured animals, it is found that in several places they contain small bodies, which are deeply coloured, irregular in shape and varying in size. In some places the bodies are big enough to block the vessel totally. If the preparations are examined in sections, the bodies are seen to be multiple thrombi, made up of more or less degenerated cells, especially red blood-corpuscles. The thrombi, which in the beginning are small and scarce, increase in number and size. Fig. 5 shows a drawing of one of the warts of fig. 4, with higher magnification. Fig. 6 gives the picture seen in sections. In the last figure the heavy black

border means epithelium; the rings indicate blood-vessels with thrombi and intact blood-corpuscles. The thrombi are chiefly found in and around the warts and groups of hypertrophic hair-follicles. Sometimes they are found elsewhere *within*, but never have they been observed in the skin *outside* the painted area. The thrombi are in many places found of sufficient size and in sufficient number to decrease the amount and the rate of the blood-flow.

FIG. 6.

CONCLUSION.

Tarring of the skin of white mice produces a marked local hyperæmia, with increased transudation. The hyperæmic condition lasts as long as the tarring is continued. The hyperæmic tissues are furnished with supernormal amounts of nourishing substances and oxygen. During this period the same tissues, especially the epithelium, become hypertrophic. The hypertrophic epithelium later forms warts and papillomas. The formation of these "benign" tumours is intimately connected with the occurrence of excessively dilated capillaries. At this point, small, multiple capillary thrombi are found, especially in and around the developing tumours. In a tissue which had been previously furnished with an increased amount of nourishing substances, and which had shown an increased rate of growth, the blood-vessels become more or less obstructed, and accordingly the supply of food and especially of oxygen correspondingly decreased. In the following period the manifest malignant tumours develop.

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SOME OBSERVATIONS BEARING ON THE SIZE OF HERPES VIRUS PARTICLES.

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THE virus of herpes, like some of the other so-called filtrable viruses, *e.g.* vaccinia, is held up to a very considerable extent by the most porous Chamberland or Berkefeld filter candles and since the virus bodies have not been observed with the microscope, it has been assumed that they are held back not by reason of their size, but because they are adsorbed by the filter or attached to tissue fragments. Recent experiments by Levaditi and Nicolau (1923) also suggest that the herpes virus particles are minute, since they were found to be capable of passing through collodion membranes which retained, completely or in part, such test materials as complement, diphtheria and tetanus toxins, and the hæmolysin in a rabbit-sheep antiserum. In view of these results of Levatidi and Nicolau, colloidin filters were tested in an attempt to obtain a sterile filtrate of herpes virus for experimental purposes, but the results obtained differed so much from those of Levaditi that ultrafiltration of this virus was studied in detail.

TECHNICAL DATA.

Virus.—Guinea-pig-skin strains of herpes virus isolated from cases of herpes labialis have been employed throughout. The strains were maintained at a more or less constant activity by frequent guinea-pig passage, the inoculations being made in the plantar epidermis. Virus was obtained from these lesions by killing the animal and cutting off the affected plantar skin—usually the whole of the pad down to the tendons—and then either:—

(1) Weighing the material, grinding with sand and suspending in a quantity of M/50 phosphate solution pH. 7·6 sufficient to give a 5% suspension. Light centrifuging (2000–3000 r.p.m. for 5–10 mins.) was employed to clarify the suspension. This form of virus will be referred to as "Pad emulsion."

(2) Or dropping the pad into a tube containing either broth or phosphate buffer and placing the tube in the ice-chest. Approximately 5·0 c.c. of broth or phosphate solution was used for each pad. As McCartney (1924) has shown, herpes virus will diffuse out from the tissue into the surrounding fluid. Even after 48 hours in the ice-chest the supernatant broth or phosphate contains a considerable quantity of virus which is not admixed with tissue fragments and only contains a comparatively small amount of guinea-pig protein. This form of virus will be referred to as "Broth or Phosphate Diffusate."

Collodion membranes.—Different collodions have been used in the making of membranes. Pyroxyllin (British Drug Houses) in varying amounts was dissolved in a mixture of 75 parts of absolute alcohol and 25 parts of ether. The addition of 10% of glacial acetic acid was used to make the membranes tougher and more elastic. Acetic collodion has also been employed, the pyroxyllin being dissolved in glacial acetic acid containing 2·5 gm. K_2CO_3 per 100 c.c. acetic acid. Membranes were made from the 4% alcohol-ether collodion by pouring an excess on to a circular glass plate, the plate being tilted in all directions to ensure an even distribution of collodion and to get rid of the excess. Evaporation was allowed to go on for 5 minutes and the membrane then immersed in water, separated from the glass plate when hardened, washed well in water and stored in water. Since each membrane was tested for its permeability to virus, protein and frequently bacteria as well, at one and the same time, it was considered unnecessary to attempt any elaborate technique to ensure the membranes being of constant thickness. With the weaker collodions, both alcohol-ether and acetic collodions, membranes were made by impregnating filter paper—Whatmans No. 1—with the collodion *in vacuo*. This was done at a negative pressure of 600–700 mm. of mercury for a period of 30 minutes, the filter paper discs then drained for one minute, immersed in water and a after a thorough washing, stored in water.

Filtration apparatus.—The small Seitz filter was used, the asbestos disc being replaced by the collodion membrane. The membrane rested on a layer of filter paper, separating it from the wire grid of the filter and was held in place by a tightly-fitting rubber washer. When the metal cylinder was well screwed down on the rubber washer a good joint was made, which did not allow of leakage between the washer and the membrane. The Seitz filter was mounted in an ordinary filter flask and filtration carried out by reducing the pressure inside the flask.

FILTRATION THROUGH COLLODION MEMBRANES.

After a few preliminary experiments with more or less impermeable membranes, it was decided to submit herpes virus to filtration through membranes made from 4% collodion in 75% alcohol and 25% ether as these allowed the passage of a certain amount of protein. It was quite obvious that should herpes virus pass through a membrane which allowed some protein to come through as well, no conclusions could be drawn as to the relative size of the virus and protein particles, unless one knew how much each had been diluted in the process of filtration. In the experiments described below this has been ascertained. Each virus, whether pad emulsion or diffusate had both

TABLE I.

Virus filtered.			Filtration.		Protein titration.									Virus titration.				
					Before filtration.					After filtration.				Before filtration.				After filtration.
Strain.	Dilution.	Amount.	Pressure.	Time.	1/1	1/5	1/10	1/20	1/100	1/1	1/5	1/10	1/20	1/20	1/100	1/500	1/1000	1/20
J. G. C.	1 in 20	3·0 c.c.	200 mm. Hg.	13 mins.		+++	++	+	±	+	±	−	−	+++	++	+	±	−
J. G. C.	1 in 30	3·0 c.c.	270 mm. Hg.	13 mins.		+++	++	+	±	+	?∓	−	−	+++	++	+	±	−
H.	1 in 30	3·5 c.c.	150 mm. Hg.	24 mins.		+++	++	+	±	∓	±	∓	?	+++	++	+	±	−

Protein titration: + + + = complete flocculation.
+ + = incomplete flocculation.
+ = fine flocculi.
$\left.\begin{matrix}\pm \\ \mp\end{matrix}\right\}$ = varying degrees of turbidity.

Virus titration: + + + = confluent vesicles.
+ + = numerous vesicles not confluent.
+ = moderate take.
± = 3–6 vesicles.
∓ = 1–3 vesicles.

TABLE II.

TABLE II.

Membrane.	Treatment.	Filtration of 1 in 100 guinea-pig serum in M/50 phosphate p.H. 7·6.									
		Pressure in mm. of Hg.	Time in mins.	Titration of protein.							
				Before filtration.						After filtration.			
				1/10	1/20	1/50	1/100	1/200	1/400	1/1	1/5	1/10	1/20
4% Alcohol 75 Ether 25	Washed in water only	170	20	+++		++	++	+	∓	∓	−	−	−
Ditto	„ „	150	31	+++		++	+	±	−	±	−	−	−
„	„ „	150	30	*++		++	+	∓	−	±	−	−	−
„	Soaked for 24 hrs. in 1/20 *G.-pig serum* Washed 1 hr.	170	15	+++		++	++	+	∓	+++	+++	+++	++
„	Soaked for 24 hrs. in 1/20 *human serum* Washed 2 hrs.	150	28	+++		++	+	±	−	+++	++	+	±
„	Soaked for 24 hrs. in 1/15 *rabbit serum* Washed 2 hrs.	150	30	*++		++	+	∓	−	++	++	+	±
„	Soaked in 1% solution of *egg albumen* (purified) Washed 2 hrs.	200	55	+++		+++	++	+	0	∓	−	−	−
„	Soaked in 1% *horse serum Albumen* 24 hrs. Washed 1 hr.	170	50	+++		++	+	±	−	±	−	−	−
„	Soaked in aq. *Colloidal Lecithin* 24 hrs. Washed 1 hr.	160	45	+++		++	+	±	−	±	−	−	−
„	Ditto.	160	45	+++		++	+	±	−	−	−	−	−
„	Soaked in aq. *Colloidal Cholesterol* Washed 1 hr.	170	25	+++		++	+	±	−	+++	++	+	±
„	Ditto.	180	39	+++		++	+	±	−	++	+	+	±

* The reading in these experiments is after hours at 37° C. only. All the other readings are those made after standing overnight in the ice-chest.

its protein and virus content titrated before and after filtration, the former titration being made by means of a guinea-pig precipitin serum.

(For the details of virus titration, see Bedson and Crawford (1927).)

4% alcohol-ether membranes.—The results obtained with these membranes are given below (Table I).

It will be seen that these membranes under the conditions of filtration allowed a certain amount of protein to pass; virus, in amounts detectable by guinea-pig inoculation, could not be demonstrated in the filtrate. It was thought that possibly very small amounts of virus passed through, so 2·0 c.c. of the filtrate obtained in the last experiment (virus H) was filtered through an 8% collodion membrane impermeable to protein. The filtration completed, the membrane was ground up in a sterile mortar with a few drops of phosphate solution and a guinea-pig inoculated with the suspension. Again a negative result was obtained showing that no virus had passed through.

4% alcohol-ether membranes with increased permeability to protein.—Although in two of the experiments detailed in Table I filtration had diluted the virus to a greater extent than the protein, it was felt that the demonstration of the greater size of the virus particles would be more conclusive if the filtration were carried out with membranes which allowed more protein to pass. This of course could be achieved with membranes made from a weaker collodion, but it was found that when 4% alcohol-ether collodion-membranes were soaked in dilute normal serum (1 in 10–20) for 24 hours and subsequently washed thoroughly, their permeability to protein was greatly increased. The observation is of interest quite apart from its practical application in this work and there is some evidence that the change in the membranes is produced by the cholesterol of the serum, as will be seen from Table II.

When tested with herpes virus these "treated" 4% membranes still held back the virus even though the protein suffered little or no dilution in the process of filtration.

It is of course possible that even in the case of these membranes permeable to protein the virus is being held up not because of its size, but because it is adsorbed. There is some evidence that the virus of foot and mouth disease carries a positive charge at pH 7·6 (Olitsky and Boëz, 1927), the reaction at which these experiments were being carried out. The membranes carry a negative charge, and assuming that herpes virus at pH 7·6 is charged positively, one would expect adsorption to take place. This possibility was tested for in the following way. Four c.c. of herpes virus (pad emulsion) diluted 1 in 20 with M/50 phosphate solution pH 7·6 was filtered through a serum-treated 4% alcohol-ether collodion membrane and the filtrate kept. The residue on the membrane was then washed up and down with 4·0 c.c. phosphate solution, 1·0 c.c. kept for titration of protein and virus and the remaining 3·0 c.c. filtered. The second filtrate was kept and the residue on the membrane was taken up in 3·0 c.c. phosphate solution. One c.c. of this 2nd residue was kept for titration as before, the remaining 2·0 c.c. filtered, the filtrate kept and the residue (3rd residue) taken up in 2·0 c.c. phosphate solution. The protein in the various residues and filtrates was then titrated and the presence of virus in the residues tested for and the degree of takes

compared with that produced by the unfiltered virus at a similar dilution (Table III).

TABLE III.

Filtration.			Titration of protein.								Presence of virus in residue. Undiluted.
Stage of experiment.	Pressure in mm. of Hg.	Time in mins.	Filtrate.				Residue.				
			1/5	1/25	1/50	1/250	1/5	1/25	1/50	1/250	
1st filtration 4·0 c.c.	150	34	++	+	±	?	++	±	?	−	++ (14 vesicles)
2nd filtration 3·0 c.c.	150	19	++	+	∓	−	+	∓	−	−	++
3rd filtration 2·0 c.c.	150	20	+	∓	−	−	±	?	−	−	++ (12 vesicles)
Unfiltered virus (1 in 20 in phosphate)											+++ Vesicles becoming confluent

This experiment not only confirms an observation made on several occasions, viz. that virus could be demonstrated in the residue left on membranes impermeable to the virus, but shows also that the virus can be washed free from the guinea-pig protein contained in the pad emulsion. Even though the virus failed to pass through a collodion membrane in virtue of its size alone, one would not expect to recover all of it in the residue; some would inevitably be lost in the manipulations necessary to the experiment. However, the degree of take given by the various residues when compared with one another and with that given by the unfiltered virus, indicate comparatively little loss in virus, and the result of the experiment suggests very strongly that the herpes virus is held up by these membranes because of its size.

3·0%, 1·5% and 1·0% alcohol-ether and acetic collodion membranes.—These membranes were found to allow the passage of increasing quantities of protein, the 1% acetic collodion membranes, for instance, reducing the titre of protein by about half. It was soon found that neither the amount of protein which appeared in the filtrate, nor the rate at which these membranes filtered at a given pressure, was any test of their being intact. Gross leakage could certainly be detected in this way but not microscopic leaks. Discordant results were obtained, the virus appearing in detectable, though small, quantities in the filtrates in an irregular manner. It was decided, therefore, to test for minute imperfections in the membranes, by seeing whether they would hold back bacteria. The membranes employed were not sterile, though with care in making and handling them they were only slightly contaminated. It was found that the few contaminating organisms could be killed by immersing the membranes for a short period (2–5 minutes) in 60% alcohol and then washing them in a series of changes of sterile distilled water to remove the alcohol. Membranes so treated proved sterile when tested culturally. Since the virus (Pad emulsion) contained bacteria, usually *Staphylococcus aureus*, it was easy to test the membranes in the process of filtering the virus. Another method employed was to filter a suspension of an

organism which could be recognized in the filtrate, *B. prodigiosus* being chosen because of its pigmented growth. When the membranes were tested by one or other of these methods, it was found that only those which allowed the passage of bacteria, let through herpes virus also. This is shown by the experiments recorded in Table IV.

TABLE IV.

Membrane.	Virus.		Pressure in mm. of Hg.	Time in mins.	Presence of virus in filtrate (undiluted)	Bacterial test of filter.
	Titre.	Quantity and dilution filtered.				
1·5% alcohol-ether collodion	1 in 500	2·0 c.c. diluted 1 in 20	100	24	–	Held back Staph. aureus.
1·0% ditto.	1 in 500	Ditto.	100	14	+	*Staph. aureus passed*
1·5% „	1 in 1000	3·0 c.c. diluted 1 in 20	100	28	–	Held back B. prodigiosus
1·0% „	Ditto.	Ditto.	100	17	–	Ditto
1·5% „	„	2·5 c.c. diluted 1 in 20	100	30	–	„
1·0% „	„	Ditto.	100	8	–	„

FILTRATION THROUGH CHAMBERLAND L FILTER CANDLES.

About this time a paper appeared by Zinsser and Fei Fang Tang (1927) in which the conclusion was come to that the virus of herpes was approximately the same size as foot and mouth virus. This was based on ultra-filtration experiments with 2·0% and 1·5% acetic-collodion membranes. They found some difficulty in filtering herpes virus through these membranes, only a certain percentage of positive results being obtained. This they attributed to the herpes virus being adherent to tissue particles. The membranes through which the virus was filtered were only tested for gross leakage by the rate at which water filtered at a given pressure. Similar membranes were tested for their permeability to particles such as collargol and colloidal As_2S_3, but no method was employed for detecting microscopic leaks in the actual membranes used to filter the herpes virus and they were therefore not in a position to say that the membranes which allowed herpes virus to pass through, would not also have allowed the colloidal As_2S_3 or even bacteria to do so. The six membranes used for the experiments given in Table IV were all prepared at the same time, from the same collodion and with identical technique, yet one of the six leaked bacteria. Further, the rate of filtration at a constant pressure—100 mm. Hg.—did not disclose which was the faulty membrane.

The ease with which foot and mouth virus passes through Berkefeld, Seitz and Chamberland L_2 and L_3 filters contrasts markedly with the difficulty experienced in obtaining an active filtrate of a herpes virus by any of these filters. This Zinsser and Fei Fang Tang would explain on the supposition that herpes virus in the form of a tissue emulsion is separated with difficulty from the tissue fragments and that it is these fragments, to which the herpes virus is adherent, which are held up. Since both viruses are most probably

intracellular parasites the same explanation should apply to both. The amount of herpes virus lost in filtration through a Chamberland L_2 filter is shown in Table V.

TABLE V.

Virus filtered.	Pressure in mm. of Hg.	Time in mins.	Titration of virus. Before filtration. 1/20	1/100	1/500	1/1000	After filtration. 1/20	1/100	1/500	Bacterial test of filter.
6·0 c.c. pad emulsion 1 in 20 in M/50 phosphate pH 7·6	200	4	+++	++	+	±	±	∓	−	Virus contained staph. aureus. 1 drop on agar slope gave growth becoming confluent. 1·0 c.c. filtrate in broth = no growth.
6·0 c.c. pad emulsion 1 in 20 in M/50 phosphate pH 7·6	200	14	++	+	±	∓	∓	−	−	Ditto.
3·0 c.c. pad emulsion 1 in 20 in M/50 phosphate pH 7·6	200	30	++	+	∓	−	−	−	−	Virus contained no bacteria. Filter tested by filtering 3·0 c.c. staph. suspension 1·0 × 10^6 per c.c. Filtrate in broth gave no growth.

CENTRIFUGATION OF HERPES VIRUS.

The results obtained both with filtration of herpes virus through collodion membranes and the ordinary bacterial filters suggested that the particles of this virus were of comparatively large dimensions and certainly larger than the virus of foot and mouth disease. It has been shown (Bedson and Maitland, 1925) that no demonstrable concentration of foot and mouth virus could be obtained by centrifugation at 5,500 r.p.m. for 2½ hours, so it was decided to submit to the test of centrifugation the conclusions arrived at as to the size of herpes virus. In this work the virus which has diffused out of guinea-pig herpetic pad lesions into broth at the temperature of the ice-chest has been used (broth diffusate). This form of virus was considered preferable for the purpose because it is free from tissue fragments and contains only a trace of guinea-pig protein. In these experiments a column of 5·0 c.c. virus was centrifuged over a layer of agar. After centrifuging the upper 0·5 c.c. was carefully pipetted off and kept = supernatant. The middle layers of fluid were then carefully withdrawn by means of a pipette and discarded. Approximately 0·5 c.c. was left at the bottom of the tube and this was washed up and down on the surface of the agar by means of a pipette. This last fraction is referred to as "deposit." The titration of the virus in the various fractions was carried out in the usual manner. The results of the experiments are given below.

TABLE VI.

Exp.	Titration of virus.															
	Before centrifugation.					After centrifugation (2-2½ hours at 5000 r.p.m.)										
						Supernatant 0·5 c.c.					Deposit.					
	1/1	1/5	1/10	1/20	1/100	1/1	1/5	1/10	1/20	1/100	1/1	1/5	1/10	1/20	1/100	1/200
1.	+++	0	++	±(6)	−	+(10)	0	−	−	−	0	0	++	+	±(4)	∓(2)
2.	+++	0	++	±(4)	−	+(7)	0	∓(1)	−	−	0	0	++	++	∓(1)	−
3.	+++	0	++	+(7)	∓(1)	++	0	+	±(4)	−	0	0	++	++	+	∓(2)
4.	++	0	+	+	∓(2)	++	0	+	∓(1)	−	0	0	+++	++	±	∓(1)
*5.	++	0	+	∓(2)	−	+	0	±(4)	−	−	0	0	+++	++	±(4)	∓(1)
6.	+++	++	±	∓(2)	−	±	∓(2)	−	−	−	0	0	0	0	0	0

* Virus diffused into M/50 phosphate. The numbers in brackets indicate the number of vesicles produced.

It is clear that the strain of herpes virus in question can be thrown down by means of the centrifuge, thus confirming the filtration experiments carried out with the same or similar strains of guinea-pig-skin herpes virus, and the conclusion come to is that herpes virus particles grown in this way at any rate, are of considerable size and probably within the limits of visibility. Work is in progress to see whether this is so or not. The divergence between the author's findings and those of Levaditi and Nicolau and Zinsser and Fei Fang Tang is somewhat difficult to account for. It has to be remembered in this respect, however, that their work was carried out with neurotropic strains of herpes—rabbit-brain strains—and it is conceivable that the herpes virus particles might vary considerably in size with the tissue in which they had multiplied. Although this possibility can not be dismissed as impossible such wide variation in size as these different findings would indicate seems improbable, and it is suggested that in the ultra-filtration experiments of Levaditi and Nicolau and Zinsser and Fei Fang Tang the passage of herpes virus was due to microscopic leaks. This source of error was not controlled in their experiments.

CONCLUSIONS.

1. The virus particles of a guinea-pig-skin strain of herpes are larger than the least dispersed of the guinea-pig serum proteins.

2. Evidence has been produced to show that herpes virus present in an emulsion of virulent guinea-pig tissues (pad emulsion) can be separated from the guinea-pig protein.

3. These strains of herpes virus will not pass through even 1 % collodion membranes.

4. Such a strain of herpes virus can be thrown down by centrifugation —5000 r.p.m. for 2–2½ hours.

5. These results suggest that the virus particles in these guinea-pig-skin strains of herpes are probably within the limits of microscopic visibility.

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REACTIONS OF, AND ABSORPTION BY, LYMPHATICS, WITH SPECIAL REFERENCE TO THOSE OF THE DIAPHRAGM.

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Of recent years it has been placed beyond doubt that the blood capillaries have the power to contract of themselves without reference to other portions of the vascular tree. The mechanism causing such contraction is still a matter of dispute, but the writer is one of those who holds the view that the contractility is a function of the endothelium. Recently Florey (1927[2]) and Carleton and Florey (1927) have demonstrated that lacteals of the squirrel, composed only of endothelium, are capable of active contraction when strongly stimulated, faradically or mechanically, but did not react to such drugs as adrenalin or pituitrin.

It became of interest, therefore, to investigate the mechanical properties of lymphatic capillaries and trunks in other portions of the body. The diaphragm is a region notoriously well supplied with lymphatics, but offers obvious difficulties for microscopic observation in the living state.

Efforts were first made to detach the diaphragm peripherally and pull a flap of it into such a position that its pleural surface could be observed, the animal being kept alive by artificial respiration. This, however, proved to involve too much manipulation of the tissues, and the rabbit, the animal used, stood artificial respiration with open thorax very badly.

The method finally adopted was as follows:

The animal—rat, rabbit or guinea-pig—was rapidly killed by a blow on

the head and the whole of the thorax removed as quickly as possible. The pleural surface of the diaphragm was thus exposed. The lower portion of the animal was then mounted, diaphragm up, in a clamp. The surface of the diaphragm was continuously irrigated with Ringer-Locke solution at 37°C.

The preparation was then ready for examination by a stereoscopic binocular microscope (Zeiss model).

These manipulations in the case of the rat and guinea-pig took about three minutes, *i. e.* from the moment of death to the actual moment of observation. In rabbits the time was usually about five minutes.

The larger lymphatic trunks on the pleural surface could be seen against the muscle background when filled only with natural lymph, but advantage was taken of the fact that it is possible to inject the lymphatics by placing suitable substances in the peritoneal cavity.

Several substances have been tried. A strong solution of hæmoglobin has been used for some observations. In others, blood concentrated by centrifuging was used. With this latter a very good picture was obtained, the lymphatics being filled with the corpuscles after an interval which was usually two hours, though in the light of later experiments this could be reduced.

Solutions of indian ink and suspensions of carmine have been tried, but, for reasons to be stated later, were found to be quite unsuitable. Cream was also tried, but gave an inconstant filling of the lymphatics with fat.

The substance, however, producing the most uniform and clear-cut pictures was hydrokollag 300, first introduced into physiology by Drinker and Churchill (1927). The ammonia in the substance as supplied was blown off with a current of air, and before injection the ink was diluted with saline. The animal preparation could be made 3–5 mins. after intraperitoneal injection. By the time the preparation had been mounted the flow of lymph had stopped, but the capillaries and trunks remained full of the injection material.

The appearances differed in the species examined. The tendinous portion of the diaphragm is relatively extensive in the rabbit. On its upper surface it exhibits a close network of tubular vessels which anastomose frequently. The small capillary vessels empty into larger trunks which course over the muscular portions.

In the guinea-pig and rat it was only the relatively large trunks which course on the muscle which were observed, the capillary arrangement of the rabbit being absent in the rat and very much reduced in the guinea-pig.

REACTION OF THE DIAPHRAGMATIC LYMPHATICS.

Mechanical and faradic stimuli were applied by means of a fine glass rod and monopolar or bipolar electrodes respectively. In observations by this means two difficulties arise. It is very easy to cause passive collapse of the vessels, and as there is no current flowing, and consequently internal pressure, there is slight tendency for recovery. After numerous observations it was decided that no reliance could be placed on results obtained from these methods of stimulation with respect to the capillary vessels on the tendinous portion of the diaphragm. The other difficulty becomes apparent when dealing with the vessels on a muscular background. The idio-muscular contraction

first described by Schiff (1858) renders the interpretation of observations difficult. This phenomenon can be excellently seen on the diaphragm. If a point is drawn sharply across the muscular part of the diaphragm the track becomes marked by a sharply defined "wheal," due to the contraction of the muscle just beneath the point of stimulus. So sharply is this contraction outlined that letters and designs can be traced on the surface. The contraction is maintained for several seconds and then gradually fades away in much the same way as smooth muscle reacts in similar circumstances. This phenomenon is quite independent of the central nervous system. The result of this localized contraction of the skeletal muscle to mechanical stimulus was to distort the overlying lymphatic trunk, so that it appeared contracted. Whether all this contraction was due to the skeletal muscle or was partly from an intrinsic contraction of the lymphatic wall could not be satisfactorily determined.

These disturbing factors, however, do not apply to the investigation of drugs. These have been applied warm from a pipette during a temporary stoppage of irrigation.

It may be briefly stated that adrenalin chloride (1/1000 and 1/10,000), pituitrin (1/100, 1/50, as supplied in capsules), pilocarpine nitrate (1/1000), barium chloride (1/200) have been found to have no action on any of the vessels with the exception of those of the guinea-pig.

In this animal, immediately after the setting up of the preparation, rhythmical, propagated contractions were observed in the trunks. These were of exactly the same nature as those described in the mesenteric lacteals of this animal (Heller, 1869; Florey, 1927[1]). The rate of beat was variable, but is usually about 15 per minute. They ceased a few minutes after observation, but then a complete beat could often be elicited by mechanical stimulation. The whole superficial network of vessels has never been seen in activity at the same time, but the active portions differ from animal to animal, so that there is no reason to suppose that any special part of the network is alone endowed with this property. Though the vessels may be rhythmically active in one portion, those inactive may be quite insensitive to mechanical stimuli.

It has been noted that in the excised mesentery of the guinea-pig the rhythmic activity of the lacteals very soon ceases, as is the case in the diaphragm. The explanation offered was that the cessation of lymph-flow eliminated the preliminary stretching of the smooth muscle-fibres of the lacteal wall which was thought to be the stimulus for contraction. The same explanation is offered for the early cessation of rhythmical activity in the vessels of the diaphragm.

The lacteals of the rat are also rhythmically contractile, but in no case have rhythmic contractions in the diaphragmatic vessels been seen. These observations, of course, do not rule out the possibility that the diaphragmatic vessels may be rhythmically contractile under more normal conditions.

The rhythmic activities of the guinea-pig's diaphragmatic lymphatics are apparently under sympathetic control, as judged by the action of adrenalin. The application of this drug increases the frequency of the beat, in one case from 15 to 24 per minute. However, when the contractions had ceased adrenalin would not cause a return of rhythmicity or spasm.

To sum up, the experiments afford no evidence of the contractility of lymphatic capillaries, but show that in one species, at least, the lymphatic trunks of the pleural surface of the diaphragm are rhythmically contractile.

THE PASSAGE OF PARTICULATE MATTER INTO THE LYMPHATICS OF THE DIAPHRAGM.

In a recent review Cunningham (1926) has stated that—"In general, then, we may summarize the work which has been done on the mechanism involved in the absorption of particulate matter from the peritoneal cavity in the following way: The earlier work all tended to establish the concept of the presence of actual pre-formed physical openings between the peritoneal cavity and the diaphragmatic lymphatics. This idea was gradually eliminated, and in its place the concept of potential physical openings between cells was offered. In turn this hypothesis is being replaced by one which assumes that most, if not all, of the particulate material that is absorbed from the peritoneal cavity passes directly through the living cytoplasm of the mesothelial cells."

The reader is referred to this article for a discussion of the evidence upon which this view is founded. An article by MacCallum (1903) also has a useful review of work done prior to 1903 on this subject. It appeared to the writer that there was probably no reason to suppose that the lymphatics of the diaphragm were possessed of particular properties, so that information obtained from observations in this region would probably be generally applicable.

The doctrine of the passage of large particles through the protoplasm of cells merely as a method of transport, on the face of it, seems improbable, so that the question was thought to be well worth re-investigation.

Experimental.—During the examination of the motor activities of the diaphragmatic lymphatics a technique was developed whereby injections of various fluids could be made into them.

A Barber micro-manipulator was mounted on a stand, giving a coarse vertical movement. Pipettes were made from hard glass tubing which was drawn to a fine point at one end, the other being cemented by means of de Khotinski's cement into a brass collar. This collar was screwed on to a brass tube connecting with a hypodermic syringe.

In making an injection the glass tube was filled with the injection material and then screwed on to the rest of the apparatus, which had previously been filled with water so as to avoid the inclusion of air-bubbles. The glass pipette was then mounted in the micro-manipulator.

The early injections were made without control of injection pressure, but it soon became evident that such was desirable. A small side-tube, to which was attached a vertical water manometer, was inserted into the brass tube connecting the hypodermic syringe and the pipette.

A freshly excised diaphragm was mounted flat on a cork attached to a heavy lead base. Hedgehog quills were used for pinning out the tissue, as these could be cut off close to the cork and so did not interfere with the pipette, which approached obliquely.

The injection was made under a binocular dissecting microscope, the oblique lighting being provided by a 100 c.p. pointolite.

The fluids used have been Indian ink, hydrokollag 300 and a solution of starch coloured with iodine. This latter solution contains, after suitable filtration, numerous small particles easily seen under the microscope, which nevertheless do not clog up the mouth of the pipette.

MacCallum also injected the peritoneal lymphatics (largely those of the dog) under a binocular microscope, but he had no control on his injection pressure nor does he state the fluids he used. He states that he was able to inject the lymphatics on the peritoneal surface fully with very little leakage. He could fill the spaces he called lacunæ without any leakage at all, but as the pressure was raised drops of clear fluid issued, and on raising it still more there was a swirl outwards of injection material, which he took to be due to rupture of the vessel wall.

The present work was done almost exclusively with the diaphragm of the rabbit and rat. The diaphragm was excised with the greatest care, and mounted, peritoneal surface up, on the cork. It was kept covered with saline throughout the experiment.

I agree with MacCallum that in certain cases all the lacunæ can be filled without evidence of escape of injection fluid. This injection has to be made with great slowness.

However, I have been surprised at the low pressure at which substances like hydrokollag and iodized starch will escape from these vessels. The lowest pressure in the rabbit at which streams of injection material were seen issuing into the overlying saline was 2·5 cm. water. At this pressure only three or four thin streams could be seen, but these increased in number as the pressure was raised till large amounts of injection material poured out from numerous points at about 30 cm. of H_2O pressure. It is to be noted that the leakage only occurs from the surface of those portions of the lymphatics in intimate contact with the peritoneal cavity (the lacunæ), and not from the vessels more deeply embedded till very high pressures are reached.

This result has been obtained very frequently, and invariably the same picture is presented. Experiments will be quoted to show that these low pressures at which material escapes are quite of a physiological order.

These injections also show, incidentally, what a very low pressure is sufficient to cause fluid to pass along these vessels.

Fig. 1 illustrates the picture left after the injection of indian ink into the lymphatics of the posterior portion of the peritoneal surface of the diaphragm of a rabbit. Fig. 2 is a preparation from a rat.

It was considered probable that pressures of this order were developed between the diaphragm and liver in quiet respiration. To test this a small rubber balloon tied on to the end of a gum-elastic catheter of small bore was inserted between the liver and diaphragm of a rabbit anæsthetized with urethane and ether through a small abdominal incision. The pressure was measured by connection to a U-tube water manometer made from thermometer tubing. After the insertion of the balloon the abdomen was closed by suture and as much air as possible evacuated from the peritoneal cavity.

Under these conditions a difference of pressure of 3–5 cm. was developed by the respiratory movements of the diaphragm. The abdominal walls were, of course, completely relaxed owing to anæsthesia, but it was seen that slight

pressure by the hand was capable of raising the pressure in the balloon several centimetres of water. One might reasonably expect that active contraction of the abdominal muscles would be capable of putting up the pressure very considerably.

It was thus that attention was focused on the importance of intra-abdominal pressure, but a digression is necessary here to consider the properties of substances commonly used previously for observations on absorption from the peritoneal cavity.

Indian ink has been frequently employed. This, as is now well known, precipitates in large masses in contact with serum. Consequently, on injection of this substance, the bulk of it is flocculated in contact with the albuminous fluids covering the peritoneum. One has only to examine an abdomen after such an injection to appreciate this fact, especially if a considerable quantity of the saline vehicle has been absorbed by the blood-vessels.

The same objection applies to suspensions of carmine. These facts can be readily appreciated by examining the lymphatics of the pleural surface of a diaphragm after injection of such substance. The material in them is seen to be clumped together, and is not uniformly spread.

It seems probable, therefore, that small pieces have to be detached by mechanical means (respiratory movement, *e.g.*) from these coagula before they can enter the lymphatics. The introduction of hydrokollag 300 into physiology by Drinker and Churchill seemed to offer a very useful substance for injection. It is a stable suspension of very fine graphite particles, which do not precipitate in the presence of serum. This expectation has been completely realized.

For use, the ammonia which the commercial preparation contains was blown off by the air, and the resulting product mixed with an equal volume of saline.

20 c.c. of this was injected into the peritoneal cavity of a 1500 gm. rabbit. From the first experiments with this, one was amazed at the speed of absorption. The first rabbit was killed 5 minutes after injection. All the diaphragmatic lymphatics were found to be evenly injected, the thoracic duct was full of black graphite ink for its whole length, and the mediastinal glands were jet black.

One can now revert to a consideration of the importance of intra-abdominal pressure in the absorption of particulate matter in suspension.

A rabbit was killed by a blow on the head, and its thorax removed so as to expose the pleural surface of the diaphragm. 20 c.c. of graphite ink were injected intraperitoneally and allowed to run between the diaphragm and liver. The intra-abdominal pressure was then raised by steadily squeezing with the hand. With this manœuvre the whole of the diaphragmatic lymphatics were rapidly and completely injected. With this evidence of the importance of intra-abdominal pressure the following experiment was done:

A rabbit was anæsthetized with urethrane (·75 c.c. 25% solution intravenously) and fixed with its pelvis well above the level of the diaphragm. Twenty c.c. graphite ink were then injected, and the animal killed quickly, without struggle, by means of chloroform at the end of three minutes. During the experiment the animal had breathed quietly and regularly.

An examination of the diaphragm showed that there was a very small collection of ink in a few lymphatics—the preparation had to be observed with the dissecting microscope before they could be seen.

Another rabbit of the same size was taken, and without anæsthesia injected intraperitoneally with 20 c.c. of graphite ink. This rabbit immediately after injection was picked up by the hind legs, so that in its efforts to right itself it utilized its abdominal muscles and so increased its intra-abdominal pressure. It was then urged to jump around the floor. At the end of 3 minutes it was killed by a blow on the head and the diaphragm examined. In this animal the injection of the diaphragmatic lymphatics was complete, and the graphite ink was issuing from the thoracic duct into the venous system. The conclusion would seem to be inevitable that the intra-abdominal pressure plays a predominant *rôle* in the removal of both fluids and particles from the abdominal cavity by way of the lymphatics. A corollary of this would naturally be that experiments performed on the relative rates of absorption by the lymphatic system and blood-vessels on anæsthetized animals give very far from an accurate picture of what occurs under normal conditions. The amount absorbed by the lymphatic system depends to a large extent on the activities of the animal.

Mode of exit of particles.—The rapidity and dependence on pressure of the processes just described would render it very unlikely that the particles passed through the cytoplasm of cells on the way out of the abdominal cavity. Phagocytic cells, once they have taken up particles, do not readily discharge them. Though the phagocytic cells take up particles rapidly, the rapidity of ingestion is not to be compared with the rapidity with which the graphite ink particles *issue from the thoracic duct.* Cunningham argues that because he finds a few avian corpuscles and particles of ink in the cytoplasm of the mesothelium and endothelium, therefore these are in transit. But these cells are apparently capable of phagocytic activity, so that to find cells and particles within them is not good evidence for transport of such *viâ* the protoplasm.

However, apart from these arguments, the histological pictures, obtained from diaphragms through which graphite ink was passing, furnish, I think, very good evidence that the earlier conception of MacCallum is correct, and not that which Cunningham wishes to substitute.

MacCallum performed the following experiment: Indian ink was placed on the diaphragm of a dead eviscerated dog and artificial respiration carried out for 2 to 3 hours. From such a diaphragm he obtained a preparation in which the mesothelial cells were roughly outlined by deposit of carbon granules. From this he came to the conclusion that some at least of the carbon passed through between the junctions of the mesothelial and endothelial cells.

Cunningham, however, in repeating these experiments, was unable to obtain this result.

The accompanying figures illustrate the appearance of the peritoneal surface of the diaphragm of the rat. This animal was injected intraperitoneally with 3 c.c. of hydrokollag 10 minutes before death. Each mesothelial cell is beautifully outlined by a dark line of carbon, and it is the mesothelial cells over the lymphatic lacunæ which appear so. Very similar

appearances are presented by the lymphatics of the rabbit, though no preparation has been obtained so beautiful as those from the rat (Figs. 3 and 4).

That the graphite is between the cell-borders in the rabbit has been shown by an impregnation with silver, when it can be seen that the carbon is distributed along the silver lines. But there is a further interesting phenomenon if the ink is left in the peritoneal cavity for 15 minutes. Examination of pieces of abdominal wall shows the mesothelial cells, in parts, faintly though completely outlined. If any graphite has passed into the subjacent lymphatics it is only a very slight amount, and would have to be sought in sections under the high-powered microscope.

The work of MacCallum provides a possible reason for the fact that cells overlying the diaphragmatic lymphatics are darkly outlined and those elsewhere only faintly. He showed that there is a very tenuous complete basement membrane to the mesothelial cells except over the diaphragmatic lacunæ, where it is replaced by a kind of fenestrated membrane. The graphite particles can pass through this fenestrated membrane but not through the complete basement membrane, at least in appreciable quantities, though the particles can penetrate between the cells as far as the basement membrane.

It is, of course, not denied that the phagocytes play a part in removing foreign material, but this is a process which appears later than that considered here.

The penetration between the mesothelial cells only has here been considered. Kolossow (1893) showed that endothelial cells of lymphatics and mesothelial (epithelial) cells are joined in an exactly comparable way, so that it may fairly be concluded that what holds for one holds for the other. The argument from the speed of the passage of the particles of graphite applies to the endothelial as well as to the mesothelial cells.

It has been noticed that once the graphite ink or other particles have become inserted into the lymphatics, they do not make their way out again through the walls of the pleural lymphatics or those connecting the peritoneal and pleural plexuses.

There are two possible explanations: one is that a pressure sufficient to force particles out between the endothelial cells is absent, or that the general lymphatic wall is less permeable than the roofs of the lacunæ in contact with the peritoneal cavity. The same phenomenon is seen during fat absorption, to be discussed more in detail later. Fatty particles enter the lacteals of the villi, but do not come out again at any point on the way to the lacteals of the mesentery. At present the writer has not sufficient evidence to settle which is the correct explanation.

The following observations indicate that colloidal solutions possibly penetrate into the lymphatic vessels through the cytoplasm as well as between the cells.

After the injection of a concentrated hæmoglobin solution into the peritoneal cavity, it was found that there was a generalized diffusion of pigment through the peritoneum, which was stained dark red. The lymphatic trunks from these areas also contained hæmoglobin. This can be especially well seen in the trunk or trunks running from the glands at the bifurcation of the aorta to

the cisterna chyli. Evidently the hæmoglobin will penetrate where particles will not.

Injections of trypan blue solution into the pleural lymphatics were made. It was found that the blue solution diffused evenly out of the vessels into the tissues and was quite useless for the purpose in view. But it can be taken as evidence that dyes will pass through the cell protoplasm. It is not, of course, suggested that in this last observation the lymphatics are in a normal condition.

The reason for the presence of this very efficient absorptive mechanism is not clear, as it would seem at first sight probable that the peritoneal cavity normally does not have to deal with particulate matter to any extent. There may, however, be a very considerable constant flow of lymph from the blood-vessels lining the peritoneal cavity to the diaphragmatic lymphatics. That this flow may reach considerable dimensions is indicated by the fact that in one animal at least—the guinea-pig—there is a pumping mechanism of the lymphatic vessels themselves.

A fact which emerges from the experiments with graphite ink is the importance of pressure in forcing the carbon particles into the lymphatic system.

As stated at first, there is no reason to regard the diaphragmatic lymph-vessels as possessed of peculiar properties.

Sabin (1916) has summarized the literature and given conclusions from her own work on the anatomy of the lymphatics. From this it is gathered that there is a unanimity of agreement among anatomists that the lymphatic system is a closed one, *i. e.* completely lined with endothelium. That being so, the same problem as to how particles and lymph get into the lymphatic system arises for other parts of the body.

For example, the central lacteal of the villus lets in fat particles. The intermediation of "leucocytes" has long been evoked (Schafer) to account for the transport of fat from the base of the epithelial cells lining the gut into the central lacteal. This view is still widely held and given credence in a quite recent review by Bloor (1922). The histological work of R. Heidenhain (1888), however, made it highly improbable that leucocytes were involved in the process, though he admitted that they could be found in the villi, and that some contained particles giving the fat reaction.

One can conceive of the central lacteal as corresponding to the diaphragmatic lacuna, and the pressure in the tissue caused by contraction of the muscle in the villi as corresponding to the intra-abdominal pressure. The mechanism of the passage of fat particles into the lacteals thus may be exactly comparable to the entrance of particles of hydrokollag into the diaphragmatic lymphatics.

There are a certain number of facts to support this conception. If a drop of lymph taken from a mesenteric lacteal of a rat or cat, previously fat fed, be examined with the dark-ground illumination, it is found to be crowded with fat particles in vigorous Brownian movement. These are also visible with careful lighting under a $\frac{1}{6}$ objective, or, better, a $\frac{1}{12}$ oil immersion. They are between 0·5 and 0·75 μ in diameter and appear to be of almost uniform size. The classical pictures, however, depict fat-globules of varying sizes in the mucosa of the intestine during fat absorption. In explanation of the latter

one may point out that if osmic acid be added to a preparation of lymph watched with a dark ground, it can be seen that there is a tendency for the fat particles to clump together, and in some instances to form larger globules.* This is strong presumptive evidence that the size of fat-globules in fixed and stained preparations is not that present in life.

It has been noted by Peyton Rous (1908) that pilocarpine may render lymph, issuing opalescent from a thoracic duct cannula, for a short time chylous, or may for a period increase the fat in one already milky. This might be interpreted as follows: The pilocarpine causes a vigorous action of the intestinal muscle, which increases the pressure in the tissue spaces around the lacteals and quickly forces into them the small amount of fat free in the tissues.

Fat absorption ceases soon after the institution of anæsthesia. A suggestion of the mechanism is that the muscular action of the gut ceases and so no fat is forced into the lacteals.

It has been noted before (Florey, 1927[1]) that red blood-corpuscles appear in the lacteals of the mesenteries of animals exposed for microscopic examination after the lapse of a certain time interval, during which, presumably, there is some inflammation of the gut and consequent diapedesis of red corpuscles. These corpuscles find their way into the lacteals just as red corpuscles placed in the abdominal cavity find their way into the diaphragmatic lymphatics.

It is very easy to inject the mesenteric lymphatics by injecting hydrokollag into the mucous membrane of the intestine. This may mean, of course, that the lymphatics are actually ruptured, but I have been surprised, nevertheless, by the ease with which the injection can be made.

The facts here gathered together, though by no means conclusive, are suggestive that fat absorption takes place in exactly the same way as particles are absorbed from the abdominal cavity.

Tissue pressure appears to be of importance in driving particles into the lymphatics in other regions of the body. The skeletal muscles are apparently unprovided with lined lymphatics, but they exist in the perimysium. In an endeavour to trace the path of the particles from muscle, the following experiment was done on a rat:

The thigh muscles were exposed and the inferior vena cava partially tied. Indian ink suspension was injected into the thigh muscles, which were then tetanized on and off at second intervals for 20 minutes, at the end of which time the glands at the bifurcation of the aorta were found to be full of carbon, and a lymph trunk running along the back of the femoral blood-vessels was also jet black. This carbon on histological examination was found to be extra-cellular.

Indian ink injected into a rat which was then kept in a confined space for 24 hours was found to be largely ingested by leucocytes—mainly polymorphonuclear—and scarcely any had reached the aortic glands.

This experiment clearly demonstrates the importance of muscular pressure in forcing particles into the perimysial lymphatics.

It would seem, therefore, that tissue pressure is of the utmost importance

* This observation was made in collaboration with Dr. H. M. Carleton.

for the penetration of particles into lymphatics. This obviously has a bearing on the spread of bacteria and tumour-cells *viâ* the lymphatic system, but further work is necessary to make the processes involved clearer.

A possible therapeutic application suggests itself from these observations.

Blood transfusion in infants *viâ* the peritoneum is coming increasingly into vogue. The absorption of the corpuscles is, of course, *viâ* the diaphragmatic lymphatics, as can be very easily seen after a peritoneal injection of blood.

The suggestion is offered that raising the intra-abdominal pressure by massage or a tight binder would greatly increase the speed of absorption of the blood.

SUMMARY.

(1) A technique is described for viewing the lymphatics of the pleural surface of the diaphragm in a living state.

(2) The effects of mechanical, electrical and drug stimulation are described. The results are equivocal or negative.

(3) Rhythmic, propagated contractions have been seen in the larger lymphatic trunks on the pleural surface of the diaphragm of the guinea-pig.

(4) Evidence is presented that intra-abdominal pressure is the factor controlling the rapid absorption of fluids and particles *viâ* the diaphragmatic lymphatics.

(5) Emphasis is laid on the speed of absorption, under suitable conditions, of graphite ink (hydrokollag 300).

(6) Histological pictures are described and figured showing the passage of graphite ink out of the peritoneal cavity between the mesothelial cells, and not through their protoplasm.

(7) Some suggestions are made regarding absorption by lymphatics of fat and particles generally.

I am very much indebted to Prof. H. M. Turnbull for his opinion of histological specimens examined in the course of the work.

The experiments were made under the auspices of the Freedom Research Fund of the London Hospital.

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EXPLANATION OF PLATE.

FIG. 1.—Peritoneal surface of portion of rabbit's diaphragm after injection of lymphatics with indian ink. The transverse vessels are the "lacunæ" of MacCallum. It is through these that particles are absorbed from the peritoneal cavity to pass into the more deeply-situated vessels shown running perpendicularly to the lacunæ. These vessels join those on the pleural surface of the diaphragm. Drawn with the aid of a dissecting microscope.

FIG. 2.—Peritoneal surface of rat's diaphragm after injection of lymphatics with indian ink. Illustrates the difference in form as contrasted with those of the posterior portion of the rabbit's diaphragm. Photograph Leitz "Micca" camera and Zeiss a_2 objective.

FIG. 3.—Microphotograph of peritoneal surface of rat's diaphragm, showing the outlining of the mesothelial cells after intraperitoneal injection of hydrokollag 300. Whole diaphragm was fixed in 10% formalin and cleared in clove oil. × 110.

FIG. 4.—High-power drawing of portion of Fig. 3. A drawing had to be made as it was impossible to photograph the specimen at this magnification owing to wrinkles. × 625.

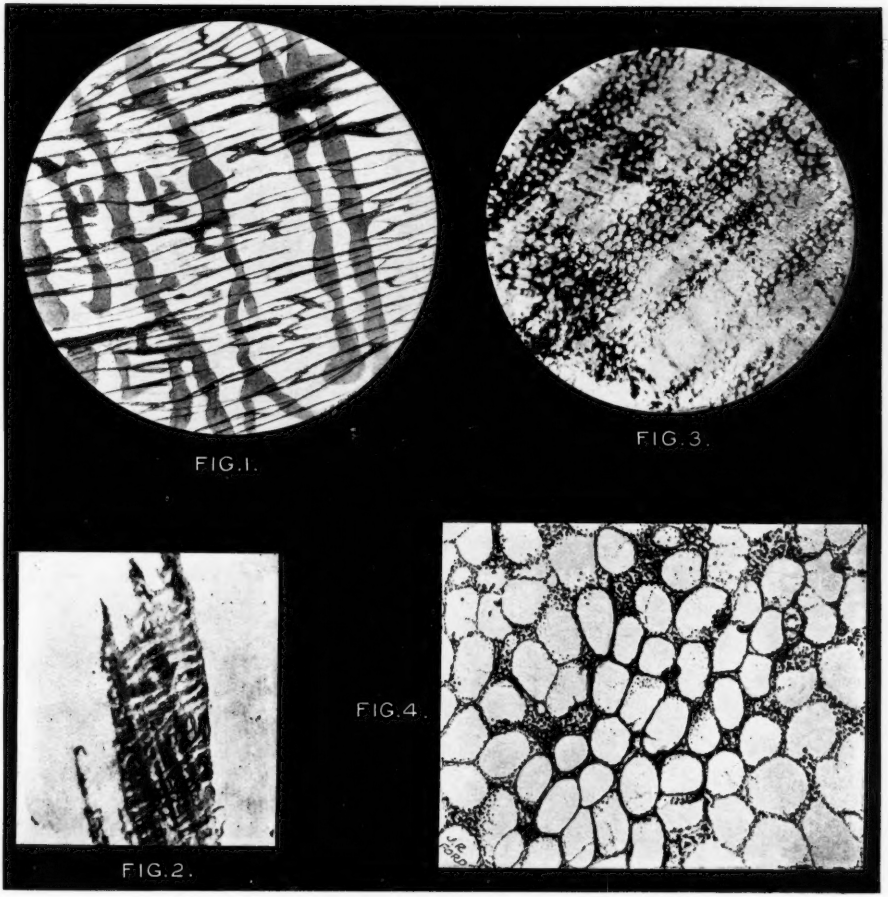

FIG. 1.

FIG. 3.

FIG. 2.

FIG. 4.

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